School of Biomedical Science

Studies on the Effect of Aging and Mesothelioma on Dendritic Cell Subsets

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

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ABSTRACT

Malignant mesothelioma is an aggressive cancer most often affecting the mesothelium surrounding the pleura. Due to a long latency period post exposure to asbestos, diagnosis often occurs in elderly individuals. However, the process of aging is associated with a functional decline in immune responses which may account for an increased susceptibility to infections, viruses and cancer. Whilst dendritic cells (DC) play a key role in generating and maintaining immune responses, including anti-cancer responses, there are conflicting results in regard to the effect of age on their number, function and phenotype, and there are no data regarding the status of DCs in elderly patients with mesothelioma. As current therapies, including surgery, chemotherapy and radiotherapy, are non-curative for mesothelioma patients, new treatments are needed. One possibility is immunotherapy, some of which target DCs.

The studies in this thesis investigated the effect of age and diagnosis of mesothelioma on DC number, phenotype and function, as well as their responses to CD40 stimulation; the latter has been used as an immunotherapeutic strategy in the clinic. Additional studies investigated the effect of direct exposure of healthy DCs from young hosts to mesothelioma tumour-derived factors. Finally, case studies are presented showing the effect of surgery and/or radiotherapy on DCs in mesothelioma patients.

Whilst $CD1c^+$ myeloid (m)DC1s and $CD141^+$ mDC2s remained stable during aging, a significant age-related decrease in $CD303^+$ plasmacytoid (p)DC numbers was observed in healthy donors aged 21 to 84 years. Monocytes from elderly volunteers differentiated into immature monocyte-derived (Mo)DCs, with lower expression levels of CD1a, CD11c, CD40, CD80, CD86 and HLA-DR relative to younger controls. Moreover, elderly-derived MoDCs showed a diminished response to stimulation with LPS and/or IFNγ. This included reduced up-regulation of the maturation marker CD83, and the incomplete loss of antigen processing implying maturational paralysis following activation. However, maturational paralysis was reversed following stimulation with CD40L, with elderly-derived MoDCs up-regulating expression of the costimulatory

molecules CD40 and CD86 and down-regulating antigen processing at higher levels than young-derived MoDCs.

Individuals with mesothelioma had significantly decreased numbers of circulating pDCs, mDC1s and mDC2s relative to their healthy age and gender matched controls. However, patients with higher mDC1 numbers than the median demonstrated a statistically significant survival advantage. In addition, MoDCs generated from people with mesothelioma expressed significantly lower levels of surface CD40 molecules and demonstrated a significantly decreased capacity to process antigen. Stimulation with LPS and/or IFNγ induced incomplete maturation indicated by a marginal loss in antigen processing capacity and an inability to up-regulate CD83 and the costimulatory molecule, CD86. Unlike healthy elderly-derived MoDCs, rescue through CD40 stimulation was unsuccessful. Exposure of healthy young-derived MoDCs to mesothelioma tumour-derived factors induced partial maturation with increased expression of CD11c, CD80 and CD86 and a muted response to stimulation with LPS or CD40L. These data imply that mesothelioma derived soluble factors permanently disable DC maturation and function.

Finally, case studies suggested that radiotherapy does not lead to a restoration of blood DC numbers or function; for example post-radiotherapy MoDCs had decreased expression of CD11c, CD83, CD86 and HLA-DR and a reduced capacity to process antigen. A further case study examined the effect of debulking surgery followed by radiotherapy. Surgery appeared to restore the phenotype and function of circulating DCs, however subsequent radiotherapy diminished this response: MoDCs demonstrated an increase in expression of CD1a, CD11c and CD80 post surgery, which was lost following radiotherapy.

In summary, these studies contribute to our understanding of the effect of age on DC subsets; in particular there is a decrease in circulating pDC numbers and maturational paralysis in MoDCs following LPS +/- IFNγ stimulation. However, CD40 stimulation rescues these DCs leading to increased CD40 and CD86 expression, which may improve

T cell responses. These studies also identified significant numerical and functional defects in DC subsets from people with mesothelioma. These results were independent of age and CD40 stimulation offered little benefit. Finally, the improved numbers and phenotype of DCs following surgery warrants further investigation and may offer a window of opportunity for the introduction of immunotherapies.

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you have been by my side for all of it. I love you with all my heart and I dedicate this work to you.

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ABBREVIATIONS

2ME	β-mercaptoethanol
APC	Allophycocyanin
APC	Antigen presenting cell
APM	Antigen processing machinery
ATP	Adenosine triphosphate
BDCA	Blood dendritic cell antigen
BSA	Bovine serum albumin
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLEC	C-type lectin receptor
CLIP	Class II-associated invariant-chain peptide
CMP	Common myeloid precursor
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T-lymphocyte-associated protein
CXCR	CXC chemokine receptor
DC	Dendritic cell
DC-LAMP	Dendritic cell lysosome-associated membrane protein
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELC	Ebstein-Barr virus-induced molecule 1 ligand chemokine
EPP	Extrapleural pneumonectomy
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor

HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HNSCC	Head and neck squamous cell carcinoma
HSC	Haemopoetic stem cells
HSP	Heat-shocked protein
IFN	Interferon
Ig	Immunoglobulin
Ii	Invarian Chatin
IL	Interleukin
iMoDCs	Immature monocyte derived dendritic cells
KIR	Killer immunonlogical-like receptor
LCs	Langerhan cells
LIN^-	Lineage negative
LPS	Lipopolysacharide
LTA	Lipoteichoic acid
M-CSF	Macrophage colony stimulating factor
mDC	Myeloid dendritic cell
MDSC	Myeloid derived suppressor cells
MFI	Mean fluorescent intensity
MHC	Major Histocompatability Complex
MIIC	MHC classII-rich endosomal compartment
MIP	Macrophage inflammatory protein
MLR	Mixed Lymphocyte Reaction
MoDC	Monocyte derived dendritic cell
NK	Natural killer
NKG2D	Natural killer group 2, member D
NKT	Natural killer T cells
NOD	Nucleotide-binding oligomerization domain
NSCLC	Non-small cell lung cancer
OVA	Ovalbumin

PAMP	Pattern-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PGE2	Prostoglandin E2
PGN	Peptidoglycan
Poly I:C	Polyinosinic-polycytidylic acid
PRR	Pathogen-recognition receptor
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted protein
ROS	Reactive oxygen species
RT	Room temperature
SEM	Standard error of the mean
SLC	Secondary lymphoid organ chemokine
SREC	Scavenger receptor expressed by endothelial cells
STAT	Signal transducer and activator of transcription
TAN	Tumour associated neutrophils
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFRSF	Tumor necrosis factor receptor superfamily
TT	Tetanus toxoid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 The Immune System

1.1.1 Innate Immunity

Outside of physical barriers, the innate immune system represents the first stage of an immune response. It responds rapidly using pathogen-associated molecular patterns (PAMPs) to recognize pathogens. PAMPs are repeating molecular structures found on pathogens and include bacterial lipopolysacharide (LPS), bacterial DNA, double stranded RNA and glucans (Aderem and Ulevitch, 2000, Akira et al., 2001, Janeway and Medzhitov, 1999). PAMPs are recognized by pattern-recognition receptors (PRRs) expressed on many immune cells including macrophages and dendritic cells (DCs) which are two key cell types belonging to the innate immune system. PRRs include Toll-like receptors (TLRs; see Table 1.1), c-type lectin receptors, NOD-like receptors and RNA-helicases. Activation of immune cells via PPRs results in an immediate response, rather than requiring proliferation first. A variety of responses occur including phagocytosis, secretion of cytokines and antimicrobial peptides and activation of the alternative complement pathway, all of which often control and eliminate an infection or contains it until the adaptive immune system is able to respond (Medzhitov and Janeway, 1997).

Table 1.1: Toll-like receptors and their ligands.

Toll-like Receptor	Ligands
1	Bacterial lipoproteins
2	LTA, PGN, bacterial lipoproteins, zymosan
3	Poly I:C, DS RNA
4	LPS, fibrinogen, heat shock proteins
5	Flagellin
6	LTA, zymosan
7	ssRNA, imidazoquinolines
8	ssRNA, imidazoquinolines

9	unmethylated CpG DNA
10	UNKNOWN
11	Profilin
12	Profilin
13	bacterial ribosomal RNA sequence

Cells of the innate immune system include macrophages, neutrophils and natural killer cells (NK). Macrophages are phagocytic cells found throughout the body which engulf and destroy pathogens. During phagocytosis they produce reactive oxygen species (ROS) which have been shown to have two functions. Firstly, ROS are cytotoxic and can act as an antimicrobial agent to aid in destroying pathogens. Secondly, ROS have been shown to act as signalling molecules in the generation of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα). Macrophages produce other proinflammatory cytokines such as Interleukin 1 (IL-1) and IL-6 which aid in recruiting granulocytes and other immune cells. Under certain circumstances macrophages can be 'switched' to a non-classical activation state where they produce anti-inflammatory cytokines such as IL-10 and tissue growth factor beta (TGFβ).

Neutrophils migrate rapidly to sites of infection or injury. Once at the site, they have various roles and can direct various immune responses. Neutrophils are also phagocytic and can internalise pathogens then destroy them with a combination of proteases and ROS. Neutrophils also generate chemoattractants and chemokines to recruit macrophages, more neutrophils and dendritic cells to the site, these factors can alter the state of recruited macrophages to either anti- or pro-inflammatory.

Natural Killer (NK) cells can be found in blood, spleen, lymph nodes and bone marrow. In the blood they make up between 10% and 20% of peripheral blood mononuclear cells (PBMC). They can differentiate between host and foreign antigens using their unique killer immunological-like receptor (KIR) which recognize Major Histocompatibility Complex (MHC) class I molecules on cells. The lack of the MHC-class I molecules on target cells induces NK cells to release perforin and granzyme to kill the target cell.

1.1.2 Adaptive Immunity

Whilst the adaptive immune response is slower than the innate immune response during the priming phase, it is more specific. It recognizes antigens via antigen-specific receptors which are encoded for by somatic recombination of gene segments. The adaptive immune system is made up of humoral and cell mediated immunity. Humoral immunity consists of B cells and immunoglobulins, whilst cell mediated immunity consists of T cells. A key component of the adaptive immune system is the generation of memory. Immune memory enables a rapid response when an antigen is recognized for the second time.

B cells have been defined as a population of lymphocytes that recognize antigenic epitopes through expression of clonally diverse cell surface immunoglobulins (Ig) (LeBien and Tedder, 2008). They are generated in the bone marrow with a potential differential end point as antibody producing plasma cells in peripheral tissues (Radbruch et al., 2006). B cells have various roles in the immune system. They are able to process and present antigen to T cells, as well as supply costimulation during presentation (Lanzavecchia, 1985). B cells can also act as immunomodulatory cells by producing cytokines that can affect T cells, DCs, regulate lymphoid tissue organization and aid in wound healing (Harris et al., 2000).

T cells play a number of roles. They are involved in providing help to other cells (such as B cells, other T cells and macrophages), recruiting granulocytes to sites of infection and inflammation, direct killing of targeted cells or pathogens and the down-regulation of immune responses. T cells can be divided into two main types, CD4 and CD8, each of which can be further divided into several subtypes.

Whilst there is a growing number of different $CD4^+$ T cell subtypes, there is a consensus as to the role of four subtypes. $CD4^+$ T helper 1 cells (T_H1) play a critical role in promoting protective immunity against intracellular pathogens. They produce cytokines such as interferon gamma (IFNγ) that enhance the generation of cytotoxic T

lymphocytes (CTL). $CD4^+$ T helper 2 cells (T_H2), instead promote immunity against extracellular pathogens and helminths. The role of the more recently described $CD4^+$ T helper 17 cells (T_H17) is still ambiguous. Studies have shown that they are capable of inducing tissue inflammation through production of cytokines such as IL-17, IL-21 and IL-22. The fourth subtype is the $CD4^+$ regulatory T cell (T_{reg}) which is further divided into naturally occurring regulatory $CD4^+$ T cells (nT_{reg}) and inducible regulatory T cells (iT_{reg}). Whilst both have different origins, with nT_{regs} produced in the thymus and iT_{regs} induced from naïve T cells in lymphoid organs and the periphery, they share common features. Both are $CD4^+CD25^+$ T cells whose key role is to negatively control immune responses and the maintain immunological tolerance (Sakaguchi, 2004).

CD8 T cells, also known as cytotoxic T lymphocytes (CTL), play an important role in adaptive immunity. When antigenic peptides expressed on MHC-class I molecules are presented to the T cell receptor (TCR) on naïve $CD8^+$ T cells, the T cell becomes a $CD8^+$ effector cell (Harty et al., 2000). The $CD8^+$ effector cell can induce the cytolysis of infected cells by two methods. Firstly, the generation of perforin and granzyme molecules by $CD8^+$ T cells leads to direct lysis of the target cell (Heusel et al., 1994, Shresta et al., 1998). The second method involves up-regulation of Fas ligand(L) which binds to the Fas receptor on the target cells and initiates programmed cell death (Ashkenazi and Dixit, 1998). Activated CD8 T cells can also secrete cytokines and chemokines that either recruit macrophages and neutrophils to the site of infection, or directly target the pathogen (Harty and Bevan, 1999).

1.2 Dendritic Cells

Dendritic cells are a heterogeneous population of cells that can take up antigen, process it and then present the processed antigen to naïve T cells to induce an immune response. They were first identified in mice by Steinman and Cohn (1973) due to their distinct morphological features consisting of a large nucleated cell with a cytoplasm that can rearrange to form varying numbers and shapes of pseudopods; their apparent lack of active endocytosis was later disproved. The lack of measured active endocytosis was due to the isolation method used at the time which inadvertently stimulated DCs, resulting in

down-regulation of their endocytic ability. DCs were first identified in humans as human leukocyte antigen (HLA)-DR^+ interstitial cells in the kidneys (Hart et al., 1981, Williams et al., 1980). Not long after DCs were characterised from human peripheral blood (Van Voorhis et al., 1982).

1.2.1 DC life cycle

As previously mentioned, DCs were first identified by their morphological features, however, recent studies using more sophisticated technology, in particular flow cytometry, have identified several DC subsets, first in mice and then in man. Whilst it is now clear that there are many subsets of DCs, each having differences in location, function and phenotype, they all share key characteristics. All DCs originate from $CD34^+$ haematopoietic progenitor cells within the bone marrow. In their immature state they have the ability to take up, degrade and present antigenic material on MHC molecules. Antigen uptake may be through receptor-mediated or non-receptor mediated methods. In response to 'danger' signals by PRR/PAMP interactions, cytokines and heat shock proteins they undergo maturation.

1.2.2 DC Subsets

DC subsets are typically classified by a number of methods including anatomical location (circulating, resident or migratory), life cycle stage (pre-DC or DC), activation state of DC (activated or unactivated) or host state (steady state or inflammatory) (Naik, 2008). This study classified DCs firstly by location then by subtype.

1.2.2.1 Circulatory DCs

In humans, circulating blood DCs make up approximately 1% of PBMCs. They are antigen presenting cells characterised by their lack of expression of typical leukocyte lineage markers (LIN^-: Table 1.2) and their high expression of MHC-class II molecules (Hart, 1997). Due the variety of markers used to identify circulatory DCs, it has been difficult to accurately define the number of subsets. In October 2010, a nomenclature was agreed upon and published for the definition of blood dendritic cells (Ziegler-

Heitbrock et al., 2010). This identified 3 subsets of dendritic cells in the blood of both humans and mice (Table 1.3).

Table 1.2: Lineage markers

Marker	Cell type	Function
CD3	T cells	T cell coreceptor
CD14	Monocytes / Macrophages	Coreceptor for LPS
CD19	B cells	B cell coreceptor
CD20	B cells	Aids in generating optimal B cell immune responses
CD56	NK cells	NK cell adhesion molecule

Table 1.3: Circulatory dendritic cells in humans and mice.

Human	Mouse
Plasmacytoid CD303^{+} blood DCs	Plasmacytoid blood DCs (CD11c^{low}CD11b^{-}CD45RA^{high})*
Myeloid CD1c^{+} blood DCs	Myeloid blood DCs (CD11c^{+}CD11b^{+}CD45RA^{-})*
Myeloid CD141^{+} blood DCs	Myeloid blood DCs (CLEC9A^{+})*

* Note: These markers are the suggested choices (until definitive makers are determined) for mouse blood DC subsets.

1.2.2.2 Myeloid Blood DCs

Within human blood, two distinct populations of myeloid blood DCs have been identified. These are known as CD1c^{+} (BDCA-1) and CD141^{+} (BDCA-3) DCs. Both express CD13 and CD33 which indicates a possible myeloid lineage. CD1c^{+} DCs (or mDC1) originate in the bone marrow, but circulate throughout the blood stream and migrate constantly to the secondary lymph organs and peripheral tissues. They comprise of approximately 0.6% of PBMCs or 19% of the HLA-DR^{+}LIN^{-} population. They express TLRs 1-8 and TLR 10.

CD141^{+} DCs (or mDC2) are a very rare population, accounting for only 0.04% of PBMCs (or 3% of the DC population). They strongly express CLEC9 and TLR3, but do

not express TLRs 4, 5 and 7 (Jongbloed et al., 2010, Huysamen et al., 2008). Apart from in the blood, $CD141^+$ DCs are also found in high numbers in the tonsils, lymph nodes, bone marrow and spleen (Galibert et al., 2005, Velasquez-Lopera et al., 2008). Due to their low numbers, their functional role is still unclear. Recent studies have shown that poly-I:C activated mDC2s induce a higher T_H1 response than mDC1s and mDC2s are more efficient in cross presentation. Most importantly, unlike mDC1s, mDC2s are able to cross present viral antigen from HCMV-infected necrotic fibroblasts (Jongbloed et al., 2010).

There is less data on blood DC lineages within mice, although there is a clear distinction between myeloid and plasmactyoid DCs. Current agreed myeloid subsets are the $CD11c^+CD11b^+CD45RA^-$ blood DCs and the $CLEC9A^+$ blood DCs (Ziegler-Heitbrock et al., 2010). The $CD11c^+CD11b^+CD45RA^-$ blood DC subset has been hypothesised to be the pre-DC population for the $CD4^-CD8^-CD11b^-$ DCs seen in mouse tissues, as they differentiate down this pathway in vitro following exposure to GM-CSF and TNF-α (O'Keeffe et al., 2003). $CLEC9A^+$ DCs in mouse blood are also $CD24^+$ identifying them as a potential precursor for $CD8^+$ splenic DCs (Caminschi et al., 2008).

1.2.2.3 Plasmacytoid DC

Human plasmacytoid DCs were originally identified as T cell associated plasma cells (Lennert and Remmele, 1958). Unlike conventional DCs, they fully develop in the bone marrow before entering the blood stream. As immature pDCs they are morphologically spherical and can be found in blood, thymus and secondary lymphoid tissue. Whilst present in peripheral tissue, they are difficult to find due to low numbers. Human pDCs are identified as $CD4^+CD45RA^+$IL-3Rα$(CD123)^+CD303^+CD11c^-$ cells. Plasmacytoid DCs use TLRs to recognize viral nucleic acid allowing them to respond to viruses without being infected. In response to a viral infection they produce large amounts of type-I interferon (IFN), which activates both innate (NK cells) and adaptive (B cells and immature DCs) immunity (Gallucci et al., 1999, Ito et al., 2001, Luft et al., 1998, Le Bon et al., 2001, Ortaldo et al., 1983). In response to PAMPs they migrate in large numbers to the site of inflammation. They can also respond rapidly to the pro-

inflammatory TLR7 ligand, imiquimod. Once activated, they adopt a typical DC morphology (i.e. dendrites) and up-regulate MHC and costimulatory molecules, although not to the same extent as conventional DCs. This up-regulation allows them to activate naive and memory T cells. Plasmacytoid DCs have also been shown to induce T cell tolerance through the induction of T_{regs} (Kuwana, 2002, Moseman et al., 2004). Freshly isolated pDCs induce anergised $CD4^+$ T cells that down-regulate IL-2 and IFNγ production and up-regulate IL-10 production. Even though pDCs can generate both priming and tolerogenic effects, it is still unclear how the outcome is determined. This may be due to pDC tissue location or maturation status. They do not seem to be involved in antigen transport to the lymph node which is predominately carried out by conventional DCs; rather, it appears that they process and present antigen at the site of infection/inflammation (Villadangos and Young, 2008). Studies claiming antigen presentation by pDCs typically only assess the T cell stimulatory/activation ability of the pDCs. These experiments measured either alloantigen or used pDCs incubated with synthetic peptides. Fonteneau et al (2003) and Salio et al (2004) have shown that pDCs have the capacity to process endogenous antigen or present exogenous peptides during viral infection. Therefore it is unknown whether pDCs are able to process exogenous antigen. pDCs have a poor macropinocytosis ability, demonstrated by their poor uptake of FITC-dextran or lucifer yellow (Ito et al., 1999, Robinson et al., 1999). However, pDCs have been shown to capably take up ovalbumin, although it is unclear whether this is via micropinocytosis or receptor-mediated endocytosis (de Heer et al., 2004, Sapoznikov et al., 2007, Young et al., 2008). Plasmacytoid DCs may also be limited in their ability to process antigen due to their low expression of cathepsin S and cathepsin D (Fiebiger et al., 2001).

1.2.2.4 Monocyte Derived DC (MoDC)

Monocytes have been characterised into two main subsets with a smaller third subset. These subsets are classical monocytes which are strongly positive for CD14 but negative for CD16; non-classical monocytes which are express low levels of CD14 and higher levels of CD16; and intermediate monocytes which express high levels of CD14 and low levels of CD16 (Ziegler-Heitbrock et al., 2010). Monocytes can be differentiated into

monocyte derived dendritic cells (MoDC) by incubation in the presence of GM-CSF and IL-4 for 5 to 7 days (Romani et al., 1994). IL-4 regulates DC differentiation by blocking macrophage development. It does this by inhibiting the production of macrophage colony stimulating factor (M-CSF), down-regulating the receptor for M-CSF and preventing loss of expression of the α–chain of GM-CSF receptor (Menetrier-Caux et al., 2001). Human MoDC differentiation typically takes longer than mouse MoDC differentiation (which can be completed in 24-48 hours). Although no proliferation occurs during differentiation, large numbers of monocytes can be obtained from a relatively small volume of blood. Differentiation generates a homogenous population of antigen presenting cells that are functionally similar to DCs. These can then be further stimulated to mature using stimuli such as LPS, TNFα, IFNγ or CD40L.

In vivo differentiation of monocytes into MoDCs appears to be limited to the generation of DCs under inflammatory conditions. Leon et al (2007) observed that during Leishmania major infection an adoptively transferred dermal monocyte population differentiated into MoDCs and was responsible for the generation of an immune response in the draining lymph node. Likewise, studies by Geissman et al (2003) and Naik et al (2006) observed that adoptively transferred monocytes differentiated into DCs under induced inflammatory conditions. These studies indicate that monocytes act as a pool of cells that can rapidly differentiate into MoDCs to aid the immune response.

1.2.2.5 Tissue Resident DCs

The examination of human DC subsets in organs and tissues other than the blood has been difficult due to lack of readily accessible material. In contrast, there has been significant effort to identify tissue DC subsets in mice. Tissue resident mouse DCs have been identified by expression of CD11c and MHC-class II, with subsets determined by expression of molecules including CD4, CD8α, CD11b, CD40 or CD205. Murine DCs were historically divided into two types, those of lymphoid lineage and those of myeloid lineage, with the lymphoid DCs expressing CD8α and myeloid DCs lacking CD8α (Ardavin et al., 1993, Wu et al., 1996, Wu et al., 1997, Wu et al., 1998). This classification was proven to be incorrect as later studies showed that $CD8\alpha^+$ and $CD8\alpha^-$

DCs could be generated by either common lymphoid progenitor or common myeloid progenitor cells (Martin et al., 2000, Traver et al., 2000).

Mouse conventional DCs are divided into subsets based on CD8α expression and tissue location. $CD8\alpha^{high}$ DCs can be found within the spleen, lymph nodes and thymus. They lack CD4 and express variable levels of CD11b and CD40. $CD8\alpha^{high}$ DCs are responsible for cross presentation of antigens to $CD8^+$ T cells (den Haan et al., 2000, Iyoda et al., 2002, Schnorrer et al., 2006), and therefore play a critical role in the induction of $CD8^+$ CTL responses to tumours and viruses (Dudziak et al., 2007, Hildner et al., 2008, Lopez-Bravo and Ardavin, 2008, Naik, 2008).

$CD8\alpha^-$ DCs are also found within the spleen and lymph node and can be further divided into $CD4^+CD8\alpha^-$ DCs and $CD4^-CD8\alpha^-$ DCs. $CD8\alpha^-$ DCs are involved in both the induction of T_H2 responses (Maldonado-Lopez et al., 1999, Pulendran et al., 1999) and the processing and presentation of antigen to $CD4^+$ T cells via the MHC-class II pathway (Dudziak et al., 2007, Pooley et al., 2001, Villadangos and Schnorrer, 2007).

Lymph nodes contain a unique population of DCs which express lower levels of CD8α. These $CD8\alpha^{int}$ DC gain access to lymph nodes via the lymphatic system and are the activated form of Langerhans cells (in peripheral lymph nodes) and interstitial DCs (in mesenteric lymph nodes).

Mouse plasmacytoid DCs have been identified in the spleen (where they make up 25% of the DC population), bone marrow, thymus and lymph nodes (Sato and Fujita, 2007). With a steady state turn-over rate of approximately 2 weeks, They are longer lasting than conventional DCs, which typically last for between 3 days and 9 days depending on location and CD8 expression (Kamath et al., 2002). In their immature form, morphologically they do not resemble a conventional DC; rather they have a rounded cell structure with only a few dendrites. Phenotypically they are described as being $CD11c^{low}$, $CD11b^-$ and $CD45RA^{high}$. Following maturation they take on typical DC morphology with increased dendrites. In response to viral infection they produce large

amounts of type I IFN. Plasmacytoid DCs have also been shown to have the capacity to differentiate into $CD11b^{+}CD45RA^{-}$ myeloid DCs.

As previously mentioned human DC subtypes have been primarily defined in the peripheral blood. Several subpopulations within tissues have been identified and described. Two major DC subpopulations reside in the skin, these are Langerhans cells and dermal DCs. Up to five subpopulations (Table 1.4) have been identified in the tonsils (Summers et al., 2001), and one in the liver (Prickett et al., 1988) and two in the thymus (Vandenabeele et al., 2001).

Table 1.4: Resident DC populations in humans

Location	Identification
Tonsil	$HLA\text{-}DR^{hi}\ CD11c^{+}$
	$HLA\text{-}DR^{med}\ CD11c^{-}\ CD123^{+}$
	$HLA\text{-}DR^{med}\ CD11c^{-}\ CD123^{-}$
	$HLA\text{-}DR^{med}\ CD11c^{+}\ CD13^{-}$
	$HLA\text{-}DR^{med}\ CD11c^{+}\ CD13^{+}$
Liver	$HLA\text{-}DR^{+}\ CD11c^{-}$
Thymus	$HLA\text{-}DR^{hi}\ CD11c^{+}\ CD11b^{-}$
	$HLA\text{-}DR^{hi}\ CD11c^{hi}\ CD11b^{+}$

1.2.2.6 Mouse DC subsets versus Human DC subsets

Several studies have attempted to correlate which mouse DC subset represents which human DC subset. Computational genome-wide expression and profiling has clustered human $CD141^{+}$ DCs (mDC2s) with mouse $CD8\alpha^{+}$ DCs, and human $CD1a^{+}$ DCs with mouse $CD8\alpha^{-}$ DCs (Robbins et al., 2008). Phenotypic similarities between human mDC2s and mouse $CD8\alpha^{+}$ DCs include expression of TLR3 (Edwards et al., 2003, Lindstedt et al., 2005) and C-type lectin CLEC9A (Caminschi et al., 2008, Huysamen et al., 2008, Sancho et al., 2008). This may have important consequences, as $CD8\alpha^{+}$ DCs in mice have a very strong capacity to cross present tumour antigens and elicit an antigen specific CTL response.

1.2.3 DC activation and maturation

The process of DC activation and maturation is usually initiated upon antigen uptake and occurs when an immature DC is stimulated by specific cytokines or via PRRs with particles such as viral RNA, or bacterial components (such as DNA or LPS). Maturation is characterized by a decrease in endocytosis and endocytic receptor expression. There is also an upregulation in expression of CD25 (IL-2 receptor), CD40, CD54 (Intracellular adhesion molecule-1), CD58 (lymphocyte function associated antigen 3), CD80 (co-stimulatory molecule B7-1), CD83 (maturation marker), and CD86 (co-stimulatory molecule B7-2). Intracellular MHC-class II molecules are loaded with antigen in lysozomes commonly known as MIICs (MHC class II containing compartments), following which they are transported to the DC surface. Morphologically the DC undergoes formation of dendrites. Following stimulation, the DC begins migration by down-regulating or internalizing tissue homing receptors (such as CCL5, CCL11 and CCL20), whilst up-regulating lymph node homing receptors (such as CCR7).

1.2.4 Antigen uptake and processing

The first stage in antigen processing is antigen capture (Figure 1.1). Immature DCs have been shown to use several methods to capture extracellular antigens. Methods such as phagocytosis and receptor-mediated endocytosis require membrane-bound receptors to internalize specifically recognized molecules. Alternatively DCs can use either macropinocytosis or micropinocytosis to internalize nonspecific molecules by extracellular fluid sampling. Phagocytosis and macropinocytosis are mechanically similar in that they both require the use of actin to restructure the plasma membrane to engulf either the particle (in the case of phagocytosis) or extracellular fluid (in the case of macropinocytosis). Neither micropinocytosis nor receptor-mediated endocytosis requires actin polymerization for vesicle formation. Rather clathrin is recruited to form pinosomes that engulf the antigenic material. Both macropinocytosis and receptor mediated endocytosis only require picomolar or nanomolar concentrations of antigen (Reviewed in Conner and Schmid, 2003).

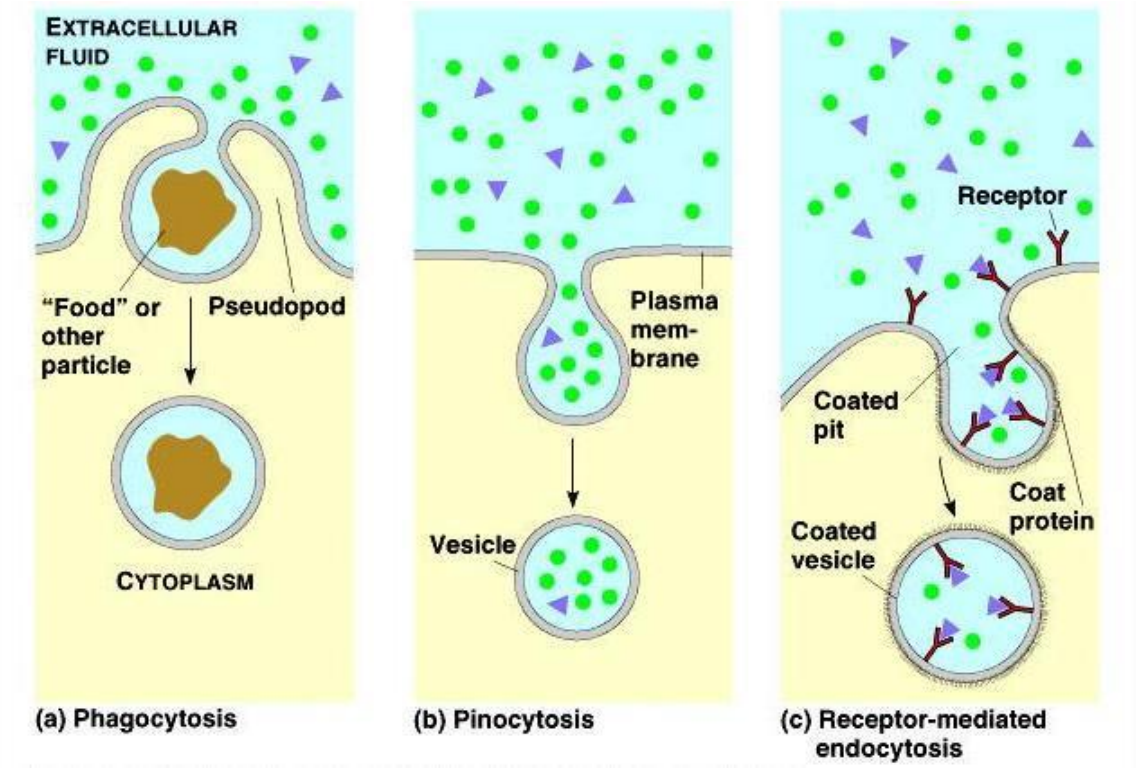

Figure 1.1: Antigen Capture (Figure from Campbell's Biology, 4th Edition (1996))

1.2.4.1 Phagocytosis

DCs phagocytose a wide variety of particulates, including bacteria, yeasts, protozoans, portions of live cells or complete or parts of dead cells. The membrane-bound receptors used by DC for phagocytosis include lectins, scavenger receptors and pathogen receptors (Banchereau et al., 2000).

Within the phagosome are various endopeptidases, exopeptidases, esterases and reductases. Whilst DCs possess a wide variety of proteases, the degree of proteolytic activity is considerably less than macrophages (Delamarre et al., 2005). The decreased proteolytic activity is due to several factors. Firstly, DCs have a reduced concentration of proteases in the phagosome (Delamarre et al., 2005). Secondly, DCs also use protease inhibitors to control the rate of proteolysis (El-Sukkari et al., 2003, Hall et al., 1998) and thirdly, the pH within the phagosome is tightly regulated, allowing the DC to activate and inactivate proteases (Jancic et al., 2007, Mantegazza et al., 2008, Savina et al., 2006). The tight regulation by DCs is used to degrade proteins down to small peptides

that can be presented on MHC molecules, rather than the total degradation seen in neutrophils and macrophages (Savina and Amigorena, 2007).

Studies have shown that DCs can phagocytose both apoptotic and necrotic cells, and then differentiate the two forms of processed antigen which may enable DCs to induce tolerance (from apoptotic) or immune (from necrotic) responses (Steinman et al., 2000). A study by Sauter et al (2000) showed that only necrotic cells or the supernatant from necrotic cells had the capacity to induce the maturation of dendritic cells. This would indicate an important role for DCs in the target and elimination of cancer.

1.2.4.2 Macropinocytosis

Unlike macrophages, which require signaling to activate macropinocytosis, this process is constitutive in immature DCs, with DCs constantly sampling the fluid environment (Norbury et al., 1997, Norbury et al., 1995, Sallusto et al., 1995, Swanson and Watts, 1995). DCs generate large pinocytic vesicles which are between 0.2 μm and 5 μm in diameter (Hewlett et al., 1994). These vesicles allow DCs to internalize large volumes of extracellular fluid containing particulate matter (Swanson and Watts, 1995). DCs are able to take up fluid equivalent to their own volume in around one hour (Sallusto et al., 1995). The excess water is then excreted via aquaporin channels in an efficient manner (de Baey and Lanzavecchia, 2000, Engel et al., 2000).

1.2.4.3 Receptor-Mediated Endocytosis

Receptor mediated endocytosis uses a wide variety of receptors which target many types of molecules, for example, Fc receptors recognize the Fc portion of immunoglobulins. Fc receptors on MoDCs include CD32 (FCγRII) and CD89 (FCαR), whilst on blood DCs they include CD32 and CD64 (FCγRI) (Fanger et al., 1996, Geissmann et al., 2001). Heat-shock proteins such as GP96 and HSP70 are taken up via receptor-mediated endocytosis via receptors such as CD91 (Basu et al., 2001). Scavenger receptors such as scavenger receptor expressed by endothelial cell-1 (SREC-1) bind modified low density lipoproteins (Berwin et al., 2004), whilst C-type lectins such as DEC-205 and DC-SIGN bind ligands such as HIV proteins in a Ca^{2+} dependent manner (Bozzacco et al., 2007,

Geijtenbeek et al., 2000). Following capture, antigens are transported to late stage endosomes, where they are proteolytically degraded.

1.2.4.4 Antigen Processing

Cytosolic proteins are the main source of endogenous antigens which are processed via the MHC class I pathway. Processing of these antigens begins with ubiquination of the proteins. This is rapidly followed with degradation by proteosomes (ATP-dependent multi-subunit proteolytic complexes). The resulting peptides are then transported across the endoplasmic reticulum (ER) membrane by the heterodimeric transporter associated with antigen processing (TAP). Within the ER the MHC-class I molecule, comprised of a heavy chain (or α chain) associated with a β_2-microgobulin chain is synthesized and immediately loaded with peptide. Proper binding of the peptide is ensured by chaperone proteins such as calnexin, calreticulin or HSP gp96. The MHC-class I/peptide complexes are then transported to the DC plasma membrane in exocytic vesicles. The overall process can be very quick as it needs to compete with rapidly replicating pathogens, and is very efficient to ensure that the DC expresses an adequate amount of epitope which has been generated by only a small amount of antigen (Guermonprez et al., 2002, Pamer and Cresswell, 1998, Savina and Amigorena, 2007).

Exogenous antigens are generally degraded via the MHC class II antigen processing pathway. This pathway begins with the synthesis of MHC-class II molecules (consisting of an α unit and a β unit) in the ER. It is here that they combine with the invariant chain (Ii). The Ii blocks the newly formed MHC-class II peptide binding site from binding self-proteins to reduce the risk of generating an autoimmune reaction. The new αβIi complex consists of 3αβ dimmers associated with an Ii trimer. The αβIi complex then travels via the golgi apparatus to the trans-golgi reticulum, where they are separated out into specialized endocytic compartments, known as the MHC-class II-rich endosomal compartment (MIIC). Within the MIIC, the Ii is partially degraded by cathepsin S to leave a small fragment known as the Class II-associated invariant-chain peptide (CLIP). The MIIC fuses with the previously mentioned phagosomes and endosomes allowing access to the degraded antigens. Finally, the CLIP molecule is removed by HLA-DM to

allow for the immediate binding of antigen peptides. The MHC molecule (with peptide attached) then travels via a transport vesicle to the surface of the DC (Tulp et al., 1994, Watts and Powis, 1999).

An alternative pathway for processing of exogenous antigens is via cross presentation. This involves transferring of exogenous antigens from the MHC class II pathway to the MHC class I pathway. Two different methods for this have been observed. Firstly antigens can escape from the phagosomes into the cytosol via the 'cytosolic pathway'. These antigens are then treated as endogenous antigens and are processed via the proteosome and TAP mechanisms. The second method known as the 'vacuolar pathway' functions independently of the proteosome and TAP components. In this pathway, antigen degradation occurs in the phagosome by endocytic proteases (such as cathepsins). The resulting peptides are then loaded onto recycled MHC class I molecules during a peptide switch in low pH endosomal compartments (Lin et al., 2008).

1.2.5 Dendritic Cell Migration

As previously mentioned, DCs are found throughout the body sampling antigens. They are also required to migrate from the site of antigen uptake to lymph nodes in order to activate T cells. Therefore the ability of a DC to migrate to the various sites within the body is a key component of the immune response. Briefly, DCs must be able to migrate (i) from the bone marrow into the blood stream, and (ii) from the blood stream into tissue sites; (iii) they also need to be able to take up residence in tissue and sample antigen; (iv) travel via either lymphatic or blood vessels to the lymph nodes; and (v) interact with immune cells within the lymph nodes. Migration of DCs is under the control of chemokines.

Immature DCs and circulating monocytes express a wide variety of tissue specific receptors which they use to migrate to parts of the body. Receptors for inflammatory chemokines (such as: CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR2, CXCR3 and CXCR4) and receptors for bacterial and complement chemoattractants (N-formyl-

methionyl-leucyl-phenylalanine-R and C5a-R) are used by immature DCs to migrate to inflammatory sites.

Once DCs enter tissues they upregulate E-cadherin to anchor themselves in place. Within the first two hours following exposure to maturation stimuli, the DC internalizes CCR1 and CCR5. This is followed by up-regulation of CCR7, whose ligands include secondary lymphoid organ chemokine (SLC) and Epstein-Barr virus-induced molecule 1 ligand chemokine (ELC) which are constitutively produced in the T cell rich zones of the spleen and lymph nodes.

1.2.6 Antigen presentation

Antigen presentation is the process by which DCs present processed antigen to naïve T cells. DCs can present antigens via MHC molecules or CD1 molecules (which is specific for lipids). The point at which DCs present antigen to T cells is known as the 'immunological synapse' (Figure 1.2). During antigen presentation three signals are used to determine the immunological outcome. As previously mentioned, following antigen capture and processing, the antigenic peptides are bound to MHC molecules on the DC surface. These antigenic fragments are stable on the surface of the DC for days, allowing time to come into contact with naïve T cells. The first signal is generated when peptide-bound MHC molecule binds to its corresponding receptor on the T cell (TCR). MHC-class II molecules bind with $CD4^+$ T cells; whilst MHC-class I molecules bind with $CD8^+$ T cells. The second signal is generated by the interaction of costimulatory molecules on the DC (such as CD80/86 or CD40) and their ligands on the T cells (CD28/CTLA4 or CD154 respectively). The role of signal 2 is to ensure that the stimulated T cell recognizes the antigen as "foreign". DCs lacking maturation stimulus, and therefore not supplying signal 2 can tolerize $CD4^+$ and $CD8^+$ T cells (inducing deletion, anergy or regulation) (Steinman et al., 2003, Steinman and Nussenzweig, 2002). T cells activated with the combination of signal 1 and 2 start producing IL-2 which leads to T cell proliferation. The interaction between CD40 and CD154 generates a feedback from the T cell to the DC which leads to increased survival in the DC, further up-regulation of MHC and costimulatory molecules and an increase in cytokine

production. The third signal is cytokine production by the DC, which determines the immunological outcome. Production of IL-12 leads to IFNγ production in T cells and polarization into T_H1 cells. This in turn leads to proliferation and differentiation of $CD8^+$ T cells into CTLs. Alternatively, the production of IL-4 skews T cell differentiation towards the T_H2 phenotype, which promote B cell proliferation, cytokine production and Ig class switching

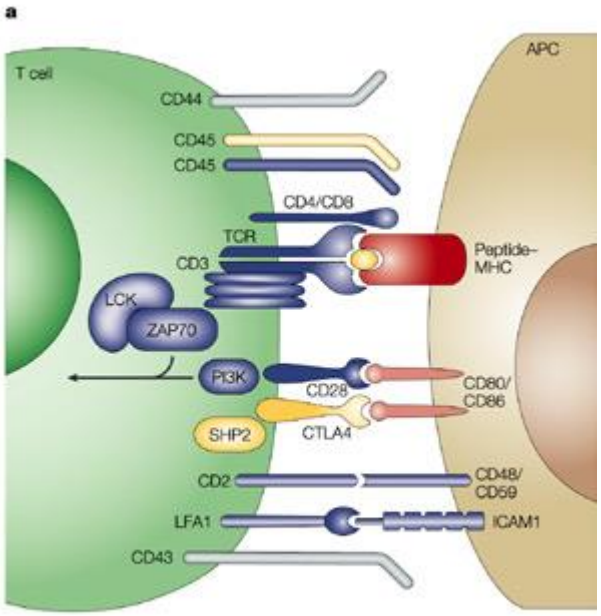

Figure 1.2: The Immunological Synapse (Figure from Huppa and Davis (2003))

1.3 Immunosenescence

Immunosenescence is characterised by a steady decrease in the ability of the immune system to respond to infection and vaccines during aging, and is believed to be the underlying reason for the increased susceptibility to cancer seen in the elderly (Larbi et al., 2008, Pawelec and Larbi, 2008). Responses to specific antigens decline, with defects in humoral and T cell responses to antigen in aged individuals reported (Weiskopf et al., 2009). T cells undergo a shift from the naive to the memory phenotype and demonstrate reduced proliferative responses and impaired cytolytic activity (Fahey et al., 2000, Rukavina et al., 1998, Walford et al., 1981). The production of high-affinity antibodies by B cells also decreases in aging hosts (Dunn-Walters and Ademokun, 2010, Frasca et al., 2011)

1.3.1 Effect of immunosenescence on the immune system (General)

The first component of the immune system affected by age is the epithelial layers of the skin, lungs and gastrointestinal tract. As the body ages, there is a breakdown in these layers allowing a greater number of pathogens to cross, placing an increased burden on the immune system (Cretel et al., 2010). However, age-related dysfunction has been observed in various components of the innate system.

1.3.1.1 Innate Immunity

Blood neutrophil numbers appear to remain constant throughout aging, and the ability of the elderly host to produce large numbers of bone marrow-derived neutrophils to fight off infections is unimpaired (Born et al., 1995, Chatta et al., 1993). However, loss of neutrophil function has been reported in aging hosts. For example, a decrease in chemotaxis, phagocytosis and production of reactive oxygen species (ROS) (Mahbub et al., 2011) has been described. Furthermore, elderly-derived neutrophils cannot be rescued from apoptosis through stimulation with pro-inflammatory cytokines (Fortin et al., 2008).

The effect of aging on macrophages is less certain. In human studies, the most conflicting problem is the definition of a ‘healthy elderly’, which depending on

screening of health can vary between publications. Secondly, most studies have used monocyte derived macrophages which may not reflect what is occurring in naturally occurring organ or tissue associated macrophages (Sebastian et al., 2005). In mouse studies, the definition of an aged mouse can vary between publications, before even taking into regard gender and species differences. Further complications can arise by different studies using macrophages harvested from different anatomical locations (Sebastian et al., 2005). As a result, the published data has conflicting results. For example, Perkins et al (1994) observed an increase in macrophage precursors in the bone marrow of aged mice, in contrast human bone marrow studies by Ogawa et al (2000) demonstrated a decrease in macrophage precursors.

Functional analysis of macrophages has also yielded conflicting results. Early studies by De la Fuente (1985, 2000) reported a decrease in aged BALB/c mice macrophages ability to adhere to tissues, their chemotaxis and to their ability to phagocytose. Others have shown either no change in phagocytosis due to age (Makinodan and Kay, 1980, Ortega et al., 1993), or an increase in phagocytic ability in macrophages from aging hosts (Kuroiwa et al., 1989, Wustrow et al., 1982). A more recent study by Ortega et al (2000) reported that the differences observed in the earlier studies were most likely due to variation in the age of the elderly population; with some studies using pre-senescent mice. Their study showed that the ability to phagocytose fluctuates throughout aging, but there is an overall trend to improve with age.

Macrophages can be divided into pro-inflammatory M1 macrophages, or anti-inflammatory M2 macrophages (Mantovani et al., 2004) and they can switch phenotypes based on the stimuli present (Arnold et al., 2007). M1 macrophages secrete pro-inflammatory cytokines, such as TNF-α and IL-12 and exert anti-tumouricidal activity (Martinez et al., 2008). M2 macrophages secrete anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β which promote angiogenesis, fibrosis and tumour growth (Martinez et al., 2008). A recent study reported that the spleens, lymph nodes and bone marrow of aged mice contain more alternatively activated M2 macrophages and myeloid derived suppressor cells (MDSC) than young mice (Jackaman et al., 2013). In vitro

experiments using peritoneal macrophages showed that elderly-derived macrophages secreted higher levels of TGF-β in response to IL-4 (a classical M2 stimulus) than young-derived macrophages. When macrophages from young versus geriatric mice were exposed to tumour (mesothelioma or lung carcinoma) cell-derived supernatants the macrophages were polarized towards the M2-phenotype regardless of age, however only geriatric-derived macrophages produced IL-4. These data imply that an immunosuppressive tumour microenvironment would be more easily formed in elderly mice. It is possible that DCs may respond in a similar manner.

In the support of a more M2 phenotype being developed with aging, murine studies show a decrease in the production of pro-inflammatory cytokines (Higashimoto et al., 1993). Specifically, a decrease in TNFα and IL-6 by both splenic and intraperitoneal macrophages following TLR stimulation is seen with age (Renshaw et al., 2002). Analysis of monocyte derived macrophages from humans has given various results. Pro-inflammatory cytokines have been observed to increase (Ershler et al., 1993, Hasegawa et al., 2000, Roubenoff et al., 1998), decrease (Beharka et al., 2001, Gon et al., 1996) or even remain constant (Ahluwalia et al., 2001) with aging. The suspected variation seen in human studies is believed to be due to changes in health status and gender in addition to differences in protocols used (Plowden et al., 2004).

Studies investigating NK cells give a more consistent image in regard to the impact of aging. In humans, there are decreasing numbers of blood $CD56^{bright}$ NK cells with increasing age, which is associated with an increase in NK cell maturity (Gayoso et al., 2011, Borrego et al., 1999, Chidrawar et al., 2006, Le Garff-Tavernier et al., 2010). Elderly-derived NK cells have reduced cytotoxic capacity on a per cell basis, with production of pro-inflammatory cytokines decreased; these include RANTES (i.e. Regulated on Activation, Normal T cell Expressed and Secreted protein), Macrophage Inflammatory Protein 1α (MIP1α), IL-8 (Shaw et al., 2010), IFNγ, TNFα, IL-2 and IL-12 (Mahbub et al., 2011).

Natural killer T cells (NKTs) have an important role in modulating immune cell responses through cytokine and chemokine production. Studies have shown various, and sometimes contradictory, age-related changes in NKTs which may lead to increased susceptibility to infection and disease. Faunce et al (2005) observed increased NKT numbers in the spleen and lymph nodes of aged mice. In contrast, DelaRosa et al (2002) and Peralbo et al (2007) reported decreased NKT cell numbers in the liver and peripheral blood of elderly humans, this was associated with an age-related decrease in NKT proliferation in response to IL-2 or α-galactosylceramide (α-GalCer) stimuli (2006). Interestingly, NKT cell cytokine secretion profile studies revealed an age-related skewing away from a T_H1 (decreased IFNγ production) and towards a T_H2 response (increased IL-4 and IL-10 production) in both basal and IL-12 stimulated NKTs from mice (Mocchegiani et al., 2009, Mocchegiani et al., 2004) as well as α-GalCer stimulated NKTs from humans (Jing et al., 2007).

1.3.1.2 Adaptive Immunity and Ageing

A number of age-associated changes have been observed for B cells and their precursors. There is a decrease in pro-B lymphocytes in mouse bone marrow, which have a diminished capacity to differentiate into pre-B lymphocytes (Cretel et al., 2010, Riley et al., 1991, Sherwood et al., 2003). Data investigating numerical changes in circulating B cells are conflicting. Cretel et al (2010) reported that overall B cell numbers remained constant, although there was an increase in $CD19^+CD27^+$ memory B cells in association with a decrease in naive B cells. In contrast, Colonna-Romano et al (2009, 2010) described an overall decrease in B cells numbers in the elderly, whilst Weksler et al (2000) reported a decrease in normal B-2 cells with an increase in B-1 cells which do not become memory cells.

Following activation, mature B cells from aged hosts have an impaired ability to class switch. Cretel et al (2010) showed an increase in Immunoglobulin (Ig) production, but a decrease in both the affinity and diversity of the antibody response. Weksler et al (2000) reported this decrease as a loss in high affinity antibodies, with an increase in auto-

antibodies. Whilst Schenkein et al (2008) observed an overall decrease in antibody potency following vaccination against Streptococcus pneumoniae.

Many studies have investigated the effect of age on the cellular arm of adaptive immunity. Thymic involution plays a key role in T cell immunosenescence. The decrease in thymic tissue leads to decreased output of naive T cells (Goronzy et al., 2007). An analysis of blood from elderly human donors showed that whilst the percentage of T cells in each T cell subset remains consistent, there is a decrease in absolute numbers of T cells relative to younger hosts (Ginaldi et al., 2001).

Apart from numerical deficiencies in T cells due to age, functional impairments have also been described. Analysis of $CD4^+$ T cell function in aged mice has shown a diminished ability to expand, differentiate and produce IL-2 upon TCR stimulation (Eaton et al., 2004, Haynes and Maue, 2009, Linton et al., 1996). Furthermore, $CD4^+$ T cells from elderly hosts have a reduced ability to efficiently form immunological synapses (Garcia and Miller, 2001, Tamir et al., 2000). More recent studies identified an age-associated skewing towards a T_H17 response following activation (Huang et al., 2008) as well as an increase in regulatory T cells in aged mice (Sharma et al., 2006, Ruby and Weinberg, 2009, Nishioka et al., 2006). Haynes and Maue (2009) postulate that the regulatory T cells consume any produced IL-2 resulting in an IL-2-deprived environment which promotes T_H17 development.

Age-associated impairments are not restricted to $CD4^+$ T cells. Bender et al (1991) and Po et al (2002) observed a reduced clearance of virus in aged mice associated with $CD8^+$ T cell impairment. In the study by Bender et al, aged BALB/C mice had a prolonged infection duration with influenza virus; the authors concluded that reduced cytotoxic T cell activity was the cause of the delayed viral clearance in elderly mice. Po et al, also observed delayed clearance of influenza virus in aged C57BL6/J mice however, this was not due to any changes to cytotoxic activity, but rather to a decrease in the number of effector T cells being produced. In a recent study by Yager et al (2008), aged C57BL6/J mice were reported to generate similar numbers of $CD8^+$ T cells to young mice in

response to influenza virus infection. However, the repertoire of response was diminished with only some viral epitopes inducing a measurable response. These data could help explain the discrepancies seen in the earlier studies.

1.3.1.3 Effect of immunosenescence on DCs

Several studies have investigated the effect of aging on DCs, but the results are contradictory. Teig et al (2002) and Orsini et al (2012) showed that blood pDC numbers in humans are highest following birth and decrease throughout adolescence. Over the same age range, mDC numbers did not change. The majority of studies investigated the effect of aging in humans from ages 18 through to between 65 and 90 years of age. Most of these studies observed that pDCs continue to decrease with age whilst mDCs remain constant (Jing et al., 2009, Orsini et al., 2012, Perez-Cabezas et al., 2007, Shodell and Siegal, 2002). In contrast Della Bella et al (2007) reported a decrease in mDC numbers with no change in pDC numbers in healthy individuals aged from 20 to 92 years old. Studies investigating the effect age has on DC numbers in animals, have been typically carried out on LCs due to the limited volume of blood able to be collected. Schwartz et al (1983) observed decreased LCs in the cheeks of elderly (78 – 80 weeks old) hamsters versus young hamsters (5 – 6 weeks old). Similarly, Choi & Sauder (1987) observed that aged mice (18 months old) had only 2/3rd the number of LCs as young mice (10 – 12 weeks old). Whilst Sprecher et al (1990) observed a steady decrease in the number of LCs as mice aged from 1 month old to 20 months old.

As blood DCs are typically difficult to isolate in large numbers, most functional human studies have been carried out on MoDCs. Whilst most of these studies (Agrawal et al., 2007, Ciaramella et al., 2011, Lung et al., 2000, Steger et al., 1996) agree that following differentiation of monocytes to immature (i)MoDCs there is no significant difference in cell surface markers due to age, Pietschmann et al (2000) observed a significant decrease in HLA-DR expression in elderly iMoDCs; it should be noted that this is the only study of this type using individuals of greater than 90 years of age. Only a few studies have investigated age-related phenotypical changes following stimulation. Neither Agrawal et al (2007) nor Ciaramella et al (2011) observed phenotypical differences to CD40, CD80,

CD83, CD86 and HLA-DR due to age following LPS activation. Similarly, Lung et al (2000) and Saurwein-Teissl et al (1998) saw similar increases in CD86 and HLA-DR expression on young and elderly-derived MoDCs following stimulation with influenza virus. Due to the limitation of blood volume in mice, in vitro functional studies are typically performed using bone marrow derived DCs. A study by Grolleau-Julius et al (2006) observed that bone marrow cells from elderly mice (21 – 24 months old) had a similar capacity to differentiate into DCs as those from young mice (3 – 6 months old).

As previously mentioned, the primary role of DCs is to sample the environment by taking up antigen, processing the antigens and presenting them to T cells. Only one study has investigated the effect of age on antigen uptake (Agrawal et al., 2007). The authors observed a significant decrease in antigen uptake with increasing age. This decrease was irrespective of whether the DC used phagocytosis, macropinocytosis or phagocytosis via the mannose-receptor. To date, no study has investigated whether antigen processing is impaired due to age.

There are limited studies investigating the effect of age on a DCs ability to induce proliferation of T cells. Steger et al (1996, 1997) observed that DCs from aged individuals (>65 y/o) maintained the capacity to induce TT specific proliferation in autologous T cells. It should be noted that the T cells used in this study were limited to (a) only being $CD4^+$ T cells, and (b) the T cells were oligoclonal T cells selected for their specific proliferative capacity. In addition, the published data only showed results from two (Steger et al., 1996) and three (Steger et al., 1997) elderly individuals respectively. A later study by the same group (Lung et al., 2000), investigated whether the previously seen proliferative capacity of elderly-derived DCs was also present in freshly isolated T cells. Whilst this study showed that elderly-derived DCs had the capacity to induce both proliferation and IFNγ production of autologous T cells, it was still limited to only showing $CD4^+$ T cell capacity and only analyzing data from a small number of individuals (n = 4). Furthermore the study did not compare the elderly-derived DCs to young-derived DCs. Alternatively, studies (unpublished) by Agrawal et al claimed reduced proliferation in young-derived T cells that had been co-cultured with

elderly-derived DCs (Agrawal and Gupta, 2011). Unfortunately, the authors did not elaborate on whether the observed reduced proliferation was in comparison to elderly-derived T cells with elderly-derived DCs, or young-derived T cells with young-derived DCs. This becomes very important, as shown in studies of DC induced T cell proliferation in mice.

Tesar et al (2006) observed that elderly and young bone marrow derived DCs induced an equal level of proliferation of allogeneic T cells. Rather any impairment due to age was associated to the T cell population, as shown when young DCs were co-cultured with both young and elderly derived T cells. Similarly, Wong et al (2010) observed no loss in T cell proliferative capacity when comparing splenic DCs from young and elderly mice. Alternativerly, Grolleau-Julius et al (2006) observed an impairment in the capacity for aged bone marrow derived DCs to induce T cell proliferation. The major difference between this study and the previously mentioned study is the ratio of DCs to T cells. Both Tesar and Wong favoured a higher DC to T cell ratio, where Grolleau-Julius favoured a high T cell population.

1.4 Cancer

Cancer is typically defined as any malignant disorder in which there is uncontrolled proliferation of cells. More specifically, six "hallmarks of cancer" have been proposed to define characteristics cells aquire before being defined as cancererous. These hallmarks are (i) the sustaining of chronic proliferation, (ii) the resisting of programmed cell death, (iii) the induction of angiogenesis, (iv) the generation of replicative immortality, (v) the activation of invasion and metastatic processes, and (vi) the evasion of growth suppressors. Recently, a 7th hallmark has been proposed, being the evasion of the immune system and subsequent destruction (Hanahan and Weinberg, 2011).

1.4.1 How cancers evade the immune system

Tumours use a variety of mechanisms to evade the immune system. These include, altering expression of surface markers, down-regulating antigen processing machinery and targeting immune cells through secretion of suppressive cytokines or expression of death receptors (Igney and Krammer, 2002).

To evade recognition by T cells, cancer cells down-regulate, internalize or shed surface MHC class I and/or class II molecules. This decreased expression has been observed in many cancers, including breast, colon, pancreatic, prostate and melanoma (Garrido et al., 1997). Total loss of surface MHC molecules does not usually occur as the tumour cells then become targets for elimination by NK cells (Ferrone and Marincola, 1995, Marincola et al., 2000). However, when total surface MHC loss does occur, NK cell function also appears to be impaired, most likely due to (i) tumour-derived cytokines (Pietra et al., 2012) and (ii) shed MHC molecules inducing NK cell apoptosis (Campoli and Ferrone, 2008).

Another mechanism cancer cells use to hide from elimination by CTLs is through defects in the antigen processing machinery (APM) (Whiteside, 2006). These defects include down-regulation of proteosomes, which lead to a decrease in the number of tumour associated antigens being produced. Secondly, variations in the proteosome subunits being formed cause changes to the characteristics of the peptides being

produced. Thirdly, cancer cells can have abnormalities in their TAP proteins which are responsible for migration of peptides into the ER (Meissner et al., 2005, Seliger et al., 2001). This leads to a decrease in stable MHC-peptide complexes being formed, as well as MHC complexes being formed which lack peptides. Lastly, changes to chaperone proteins can alter the assembly of the MHC-peptide complexes leading to their instability (Leone et al., 2013).

One mechanism tumour cells use to avoid destruction by the immune system is by targeting attacking immune cells for destruction by apoptosis. Fas ligand (FasL), a member of the TNF family (Suda et al., 1993), is expressed on tumour cells in many cancers. These include colorectal carcinoma (O'Connell et al., 1998), hepatocellular carcinoma (Strand et al., 1996), melanoma (Hahne et al., 1996) and lung cancer (Niehans et al., 1997). FasL interacts with Fas (CD95) which is predominately expressed on activated T cells and NK cells (Suda et al., 1995). The interaction of Fas with its ligand leads to a cascade of intracellular pathways that induces apoptosis in the immune cells (Itoh et al., 1991, Trauth et al., 1989, Yonehara et al., 1989).

1.4.2 Tumour derived factors

Tumours produce a range of factors (such as cytokines) that down-regulate specific components of the immune system. These factors include Vascular Endothelial Growth Factor (VEGF), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Transforming Growth Factor Beta (TGFβ) and gangliosides.

1.4.2.1 Granulocyte Macrophage Colony Stimulating Factor

GM-CSF has been shown to be produced by a large number of tumour cell lines (Bronte et al., 1999) and many cancers (Lang et al., 1994, Mattei et al., 1994, Pisa et al., 1992, Sawyers et al., 1992). Charyulu et al (2000) and Sabatini et al (1990) observed that GM-CSF production by murine and human tumours induced IL-6 and TNFα secretion which induced chronic inflammation and promoted tumour progression. Fu et al (1991, 1992) and Sotomayor et al (1990) reported that tumour-derived GM-CSF interfered with

leukocyte migration by regulating the ligands on endothelial cell responsible for leukocyte binding, blocked macrophage cytotoxicity and reduced mitogenic responses in lymphocytes. Murine studies have shown that chronic secretion of GM-CSF is associated with the induction and proliferation of myeloid derived suppressor cells (MDSCs) which suppress antigen specific T cell responses (Bronte et al., 1999, Kusmartsev et al., 2005, Watson et al., 1991).

It should be noted that GM-CSF is required for the differentiation of DCs from myeloid precursors (Figure 1.3). In steady state conditions, GM-CSF is involved in the development of migratory DCs, whilst during inflammation GM-CSF promotes the differentiation of common myeloid precursors (CMP) into precursor DCs and even splenic DCs (van de Laar et al., 2012, Naik et al., 2006).

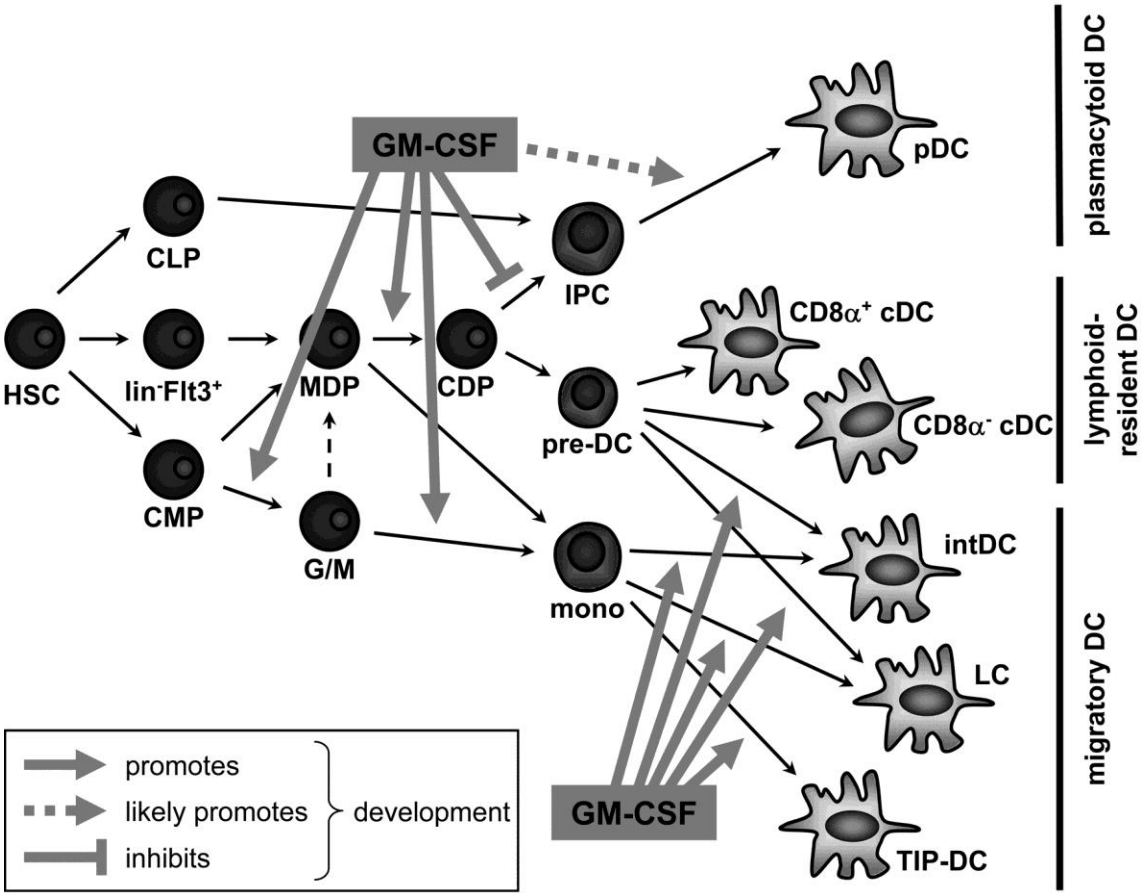

Figure 1.3: The effect of GM-CSF on DC precursor cells (Figure from van de Laar et al (2012))

The capacity for GM-CSF to promote DC development led to investigations into its use as an immunotherapy (Dranoff, 2002, Waller, 2007). The current inability to confidently target GM-CSF to specific cells such as DCs in-vivo renders it impossible to balance anti-tumour versus pro-tumour GM-CSF induced effects. Indeed, increased concentrations of GM-CSF are likely to induce the expansion of pro-tumourigenic MDSCs which promote local regulatory T cell numbers and function (Serafini et al., 2004b, Vasu et al., 2003). This leads to the generation of tolerogenic DC. Serafini et al (2004a) concluded that the amount of local GM-CSF generated has a direct bearing on whether the outcome was suppressive or not.

1.4.2.2 Vascular Endothelial Growth Factor

VEGF is produced by most tumours and is essential for tumour vasculature development. Increased levels of VEGF have been associated with a poor prognosis in cancers such as breast cancer (Toi et al., 1996), lung cancer (Salven et al., 1998), colorectal cancer (Ishigami et al., 1998) and mesothelioma (Hirayama et al., 2011). VEGF was the first tumour derived factor shown to inhibit DC differentiation. Gabrilovich et at (1996) observed that dendritic cells generated from $CD34^+$ haemopoetic stem cells were impaired when cultured in the presence of tumour supernatant produced by either breast or colon cancer cells. These impairments included morphological differences, reduced antigen uptake and low expression of surface MHC class II molecules. By using blocking antibodies, the study ruled out TGFβ, IL-10 and c-kit ligand being responsible for the impairments. Culturing the same DCs with recombinant VEGF yielded similar results, leading the authors to conclude that the DC impairment was due to VEGF. This observation was confirmed in vivo when the addition of VEGF into mice by an implanted osmotic pump led to an inhibition of DC development and an accumulation of immature DCs (Gabrilovich et al., 1998). Furthermore, use of an anti-VEGF antibody in tumour bearing mice revealed improved DC differentiation and increased numbers of DCs migrating to the spleen and lymph nodes (Ishida et al., 1998, Gabrilovich et al., 1999).

Analysis of serum from patients with gastric carcinoma showed a correlation between increased VEGF concentrations and reduced numbers of DCs in tumour tissues and peripheral blood (Takahashi et al., 2004, Saito et al., 1998). Similarly, Lissoni et al (2001) reported that a significant increase in serum VEGF concentration was inversely correlated with decreased circulating mDCs and pDCs in patients with metastatic gastric, colorectal, pancreatic, breast or lung carcinoma. In addition, Almand et al (2000) observed that an increase in VEGF levels in patients with breast, head and neck or lung cancer was associated with increased numbers of immature DCs which have a suppressive phenotype.

1.4.2.3 Interleukin-6

Increased IL-6 concentrations have been identified in the serum of patients with cancers such as ovarian cancer (Moradi et al., 1993, Plante et al., 1994), breast cancer (Zhang and Adachi, 1999), prostate cancer (Drachenberg et al., 1999) and colorectal cancer (Ueda et al., 1994). In addition, studies by Moradi et al (1993), Chung and Chang (2003) and Zhang and Adachi (1999) correlated serum IL-6 with increased tumour burden and/or presence of metastatic tumours. High levels of IL-6 have also been correlated with poor prognosis in patients with renal cell carcinoma, melanoma and colorectal cancer (Blay et al., 1992, Deehan et al., 1994, Ratta et al., 2002, Tartour et al., 1994).

Menetrier-Caux et al (1998) showed that renal cell carcinoma-derived conditioned media inhibited DC differentiation from $CD34^+$ cells, rather than switching differentiation to the generation of monocytes. Use of recombinant IL-6 in place of the renal cell carcinoma-derived media produced similar results, and blocking IL-6 using neutralizing antibodies abolished the impact of both recombinant IL-6 and the carcinoma-derived media. Similarly, sera from bone marrow aspirates containing high levels of IL-6 from multiple myeloma patients inhibited ex-vivo DC development (Hayashi et al., 2003); indicated by high expression levels of the monocyte marker, CD14, and low expression levels of the DC markers CD1a, CD80 and CD83, as well as a poor capacity to induce T cell proliferation. Blocking IL-6 restored DC development.

In addition to impairing DC differentiation from their precursors, IL-6 also interferes with DC maturation. DCs exposed to IL-6 during maturation with LPS expressed decreased levels of CD40, CD80, CD86, HLA-DR and secreted lower levels of IL-12p70 (Park et al., 2004). STAT3 knockdown experiments confirmed that IL-6-mediated suppression is through STAT3 activation.

1.4.2.4 Interleukin-10

Interleukin-10 is produced by many cancer cells including melanoma, myeloma and lung carcinoma cells (Gu et al., 1996, Kruger-Krasagakes et al., 1994, Smith et al., 1994). Whilst this accounts for some of the IL-10 seen in the tumour microenvironment, the majority of IL-10 is probably produced by tumour associated macrophages (TAM) and some tumour infiltrating lymphocytes (Seo et al., 2001, Sica et al., 2000).

IL-10 was originally shown to be a T_H2 produced cytokine with the ability to suppress cytokine production by T_H1 cells (Fiorentino et al., 1989). Further studies have since shown that IL-10 is produced by a wide variety of immune cells, including macrophages, DCs, T_{regs} and T_H17 cells (Saraiva and O'Garra, 2010). IL-10 has also been shown to reduce surface MHC class II expression on monocytes, reduce costimulatory molecules on monocytes and macrophages and down-regulate pro-inflammatory cytokine production by macrophages (Bogdan et al., 1991, de Waal Malefyt et al., 1991, Ding et al., 1993, Ding and Shevach, 1992, Fiorentino et al., 1991).

Interleukin-10 also exerts a variety of effects on DCs. The addition of IL-10 whilst culturing Langerhan cells suppressed the normal up-regulation of CD80 (Ozawa et al., 1996). MoDCs matured in the presence of IL-10 demonstrated reduced expression of co-stimulatory molecules, and a decreased capacity to induce T cells proliferation relative to the controls (Steinbrink et al., 1997, Steinbrink et al., 2002). Furthermore, pre-conditioning of MoDCs with IL-10 led to the induction of a tolerogenic phenotype which induced antigen-specific anergy in $CD4^+$ T cells.

In vivo studies showed that DCs from IL-10 over-expressing transgenic mice had a decreased ability to stimulate T cell proliferation and CTL responses and secreted less IL-12 than DCs from control mice (Sharma et al., 1999). Similarly, in a murine bladder cancer model, tumour-associated IL-10 reduced the capacity of splenic DCs to stimulate T cell proliferation and generate CTLs (Yang and Lattime, 2003). Increased IL-10 serum levels in hepatocellular carcinoma patients was associated with decreased circulating pDCs, mDC1s and mDC2s (Beckebaum et al., 2004). Furthermore, analysis of circulating DCs revealed a lack of activated mature cells, implying functional impairment due to a suppressive environment.

1.4.2.5 Transforming Growth Factor Beta

TGFβ is an immunosuppressive cytokine that affects many immune cells. It can induce cellular proliferation, differentiation and apoptosis. In the tumour microenvironment, the source of TGFβ has been shown to be tumour cells and TAMs, which make up a large percentage of the tumour mass (Mantovani et al., 1992, Pollard, 2004). Macrophages are also transformed by TGFβ. Gong et al (2012) showed that TGFβ polarized TAMs from an M1 to M2 phenotype, most likely through alterations in TβRII signaling. It is also possible that the recruitment of highly phagocytic macrophages by TGFβ leads to an overall decrease in DC effectiveness, as macrophages compete with DCs for tumour antigen (Byrne et al., 2008).

During tumour development, the release of TGFβ regulates the expression of adhesion molecules, which promote the migration of neutrophils to the tumour (Allen et al., 2008). The presence of neutrophils within the tumour microenvironment has been associated with tumour angiogenesis and metastasis (Nozawa et al., 2006, Tazawa et al., 2003). More recently, tumour associated neutrophils (TAN) have been characterized similarly to macrophages with N1 and N2 phenotypes, with TGFβ implicated as the cytokine responsible for polarization to the N2 phenotype (Fridlender et al., 2009). Apart from polarization, it has been suggested that TGFβ could affect neutrophil activity by inhibiting cytotoxicity (Shen et al., 2007).

Rook et al (1986) showed that TGFβ suppresses the lytic function of NK cells as well as their ability to produce IFNγ which stimulates CD4 T_H1 cells. This decrease is due to a down-regulation of natural killer group 2, member D (NKG2D) which is required for NK cell activation (Crane et al., 2010, Kopp et al., 2009, Lee et al., 2004).

TGFβ also impacts on T cells. For example, $CD8^+$ T cell effector function is dampened due to TGFβ signaling suppressing the genes which encode granzyme A, granzyme B, IFNγ and FAS ligand (Thomas and Massague, 2005). TGFβ promotes the differentiation of naïve CD4 T cells into T_{regs} or T_H17 cells by inhibiting T-bet and GATA3 which control T_H1/T_H2 differentiation (Gorelik et al., 2002, Gorelik et al., 2000) and by promoting Foxp3 or RORγt which induce T_{reg} and T_H17 differentiation respectively (Zhou et al., 2008).

TGFβ exerts multiple effects on DCs. TGFβ can inhibit the migration of antigen-laden DCs to lymph nodes (Imai et al., 2012, Weber et al., 2005). A decrease in DC numbers in the draining lymph nodes of lung cancer patients was associated with increased levels of TGFβ (Ito et al., 2006). In vitro experiments showed that TGFβ induced apoptosis in both cultured DCs as well as lymph node isolated DCs, leading the authors to speculate that TGFβ induced apoptosis in the lymph nodes as well, therefore aiding metastasis to the lymph node.

Whilst low levels of TGFβ induces DC maturation, high levels decrease or inhibit the expression of MHC class II, costimulatory molecules (CD80 and CD86) and maturation markers (CD83) during differentiation and maturation (Geissmann et al., 1998, Geissmann et al., 1999). These DCs (termed tolerogenic DCs) induce T_{reg} cells rather than effector T cells (Roncarolo et al., 2001). This was confirmed by adoptively transferring TGFβ-treated DCs into mice to induce T_{regs} (Alard et al., 2004). Furthermore, DCs respond to TGFβ by becoming TGFβ producing DCs, which induce T_{reg} cells (Dumitriu et al., 2009, Ghiringhelli et al., 2005).

1.4.2.6 Gangliosides

Gangliosides are composed of glycosphingolipids containing sialic-acid molecules and are involved in regulating the proliferation and differentiation of cells (Hakomori, 2003). Most tumour cells have been shown to shed gangliosides from their membrane (Bernhard et al., 1989, Ladisch et al., 1983, Ladisch and Wu, 1985, Shaposhnikova et al., 1984). Some gangliosides, such as GD3 in melanoma cells, are specific to the tumour and are not found in healthy tissues (Ritter and Livingston, 1991). They have been shown to polarise naïve T cells away from differentiating into a T_H2 phenotype by binding to IL-4. Gangliosides can simultaneously bind CD3, blocking T cell activation (Chu and Sharom, 1995, Offner et al., 1987), and bind IL-2, blocking its availability for T cell proliferation (Chu and Sharom, 1990, Irani, 1998, Lu and Sharom, 1995).

The glioma gangliosides, GM2 and GM3, can inhibit NK cell cytotoxicity (Grayson and Ladisch, 1992), The same gangliosides inhibit B cell function, by reducing TNFα and Ig production (Kimata and Yoshida, 1994). Likewise, these gangliosides inhibited TNFα gene expression in monocytes (Ziegler-Heitbrock et al., 1992).

Tumour-derived gangliosides have been shown to inhibit various components of DC development and function. Shurin et al (2001b) observed that neuroblastoma cell line-derived gangliosides inhibited the generation of DC from both mouse bone marrow progenitors and human $CD34^+$ precursors. Defects included a decrease in dendrite formation, decreased expression of CD83 and CD86 and a reduced capacity to induce T cell proliferation. Similarly, Peguet-Navarro et al (2003) reported that the melanoma purified gangliosides, GM3 and GD3, inhibited differentiation of human MoDCs including decreased expression of CD1a, CD40 and CD80, a decreased ability to induce T cell proliferation and a decrease in IL-12 production with a corresponding increase in production of immunosuppressive IL-10.

1.4.3 The effect of cancer on DCs

Several studies have investigated the effect of cancer on DCs. A decrease in circulating DCs has been observed in breast cancer, lung cancer, head and neck squamous cell

carcinoma, renal cell carcinoma and prostate adenocarcinoma (Almand et al., 2000, Gigante et al., 2009, Pinzon-Charry et al., 2007, Sakakura et al., 2006, Sciarra et al., 2007). There are several theories to account for this observed decrease. The first being an increase in apoptosis of circulating DCs. Pinzon-Charry et al (2006), showed that patients with breast cancer had a significantly higher number of peripheral blood DCs undergoing apoptosis measured by Annexin V and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Co-culturing breast cancer cells with healthy blood DCs, revealed that tumour derived factors, in particular IL-10, gangliosides and/or ceramides, were responsible for the apoptosis. Similarly, Ito et al (2006), reported an increase in DC apoptosis in the draining lymph nodes of patients with non-small cell lung cancer (NSCLC). The increased apoptosis correlated with a significant increase in tumour-derived TGFβ.

The second theory is that the decrease in circulatory cells is an indication of DCs migrating to the tumour site. Gigante et al (2009), reported that a significant decrease in circulating blood DCs in renal cell carcinoma patients correlated with a significant increase in DCs at the tumour site. In contrast, Perrot et al (2007), showed that DCs make up 1% of cells at the tumour site of patients with NSCLC which is less than the numbers reported for healthy lung tissue (3% of cells) (Demedts et al., 2005)). However, a direct comparison of the two studies is difficult as there are many differences in the source of lung tissue, tissue processing to isolate cells, the staining panels used to identify DCs and there is no data on the age or well-being of the tissue donors.

The third theory is that the decreased number of circulating DCs is due to a defect in the differentiation of DCs from $CD34^{+}$ progenitor cells. Whilst patients with lung cancer or head and neck squamous cell carcinoma (HNSCC) have a decrease in peripheral blood DCs there is a related increase in immature myeloid cells in their blood (Almand et al., 2000, Almand et al., 2001). These cells comprised of immature monocytes, macrophages and DCs as well as cells possibly at an earlier stage of development.

Phenotypic impairments of DCs in cancer patients have also been observed. Expression of co-stimulatory and MHC molecules is crucial for T cell activation, yet DCs isolated from many cancers have been shown to have reduced expression of these molecules. Gabrilovich et al (1997) showed that blood DCs isolated from patients with breast cancer had lower expression of CD80 and CD86 as well as HLA-DR relative to DCs isolated from healthy controls. Similarly, Pinzon-Charry et al (2007) reported decreased CD80 expression on blood DCs from breast cancer patients, which became more severe as the stage of disease increased. This decrease is not limited to breast cancer, decreased expression of HLA-DR on circulating DCs has been observed in HNSCC (Hoffmann et al., 2002, Sakakura et al., 2006) and ovarian cancer patients (Melichar et al., 1998).

Changes to DCs appear to become more severe within the tumour microenvironment. Nestle et al (1997) showed that only 5 – 10% of DCs in tissue surrounding basal cell carcinomas expressed CD80 or CD86 using immunohistochemistry. This number was dropped to 1 – 2% within the tumour. Similarly, Bergeron et al (2006) observed that mDCs within lung cancers did not express CD40, CD80 or CD86. In addition, Gigante et al (2009) reported that whilst the number of mDCs within the tumour increased, these DCs lacked expression of the maturation marker CD208, a DC lysosomal associated membrane protein.

Analysis of studies using DCs isolated from cancer patients concur that these DCs are impaired in their ability to induce T cell proliferation. Almand et al (2000) observed that DCs isolated from HNSCC patients had a significantly reduced capacity to induce both allogeneic T cell proliferation (measured using a mixed lymphocyte reaction) and antigen specific autologous T cell proliferation (measured using Tetanus Toxoid) compared with DCs isolated from healthy controls. Similarly, Perrot et al (2007) showed that DCs isolated from lung cancer tumours had a reduced capacity to induce allogeneic T cell proliferation relative to myeloid DCs isolated from the blood of healthy controls.

Due to the low number of circulating DCs in cancer patients, research addressing DC function is often conducted using MoDCs. MoDCS generated from patients with breast,

colon, stomach, ovarian, lung cancer and multiple myeloma do not mature as their healthy controls. Pinzon-Charry et al (2007) showed decreased capacity to up-regulate CD80 and HLA-DR following maturation with Poly I:C, CD40L or a cytokine cocktail (IL-1β, IL-6, TNFα and PGE_2). Similarly, Onishi et al (2002) described a decreased capacity to up-regulate CD80 following maturation with a different cytokine cocktail (IL-1β, GM-CSF, TNFα and IFNγ). In contrast, Harrison et al (2008) reported an increase in upregulation of CD83 and CD86 expression in myeloma patient-derived MoDCs matured with TNFα and PGE_2. It is not clear if this difference is due to the systemic nature of myeloma in comparison to solid tumours investigated in the other studies.

The effect of cancer on DC antigen uptake appears to be even more contradictory. Pinzon-Charry et al (2007) showed that MoDCs from breast cancer patients had decreased antigen uptake capacity relative to healthy age and gender-matched controls irrespective of using soluble antigen (FITC-TT) or particulate antigen (FITC-Dextran). In contrast, Ninomaya et al (1999) reported that particulate antigen (FITC-Dextran) uptake was increased in MoDCs from hepatocellular carcinoma patients.

Similar to the above results using circulating DCs, MoDCs from cancer patients also appear to be impaired in their ability to induce T cell proliferation relative to healthy controls. Pinzon-Charry et al (2007) showed that MoDCs generated from breast cancer patients had a decreased ability to induce allogeneic T cell proliferation which further decreased as the stage of the disease progressed. Similarly, Ma et al (2009) observed that MoDCs generated from HNSCC patients had a decreased ability to induce antigen specific autologous T cell proliferation.

1.4.4 Mesothelioma

Mesothelioma is an aggressive cancer of the mesothelial linings around organs and is caused by asbestos exposure. It is most typically seen (70 to 80% of cases) surrounding the lung cavity (pleural mesothelioma) (Suzuki, 2001). The remaining cases are diagnosed in the tissue around the peritoneal cavity (peritoneal mesothelioma), heart

(pericardial mesothelioma) and the tunica vaginalis of the testis (Mak et al., 2004). Mesothelioma is primarily a disease of the elderly with studies showing the highest percentile age group being those aged 60 – 69 years of age, and 64% of those diagnosed being 60 years of age or older (Suzuki, 2001). This is due to the long latency from exposure to asbestos to diagnosis (Lanphear and Buncher, 1992). Mesothelioma is predominately a disease associated with males making up 80% of those diagnosed (Robinson et al., 2005), but this is primarily due to males having the highest incidence of asbestos exposure. There is currently no cure for mesothelioma and the median survival for patients varies from approximately 8 to 14 months from diagnosis (British Thoracic Society Standards of Care, 2007). Poor prognostic factors include (i) male gender, (ii) age over 75 years, and (iii) non-epithelioid histology (Moore et al., 2008).

High incidence rates for mesothelioma are quite rare. Exceptions to this include the residents and workers from the town of Wittenoom in Western Australia. Due to heavy mining of asbestos at Wittenoom, the incidence of mesothelioma increased from 8 to 45 per million between 1972 and 1990. In this population, there has been a recorded 315 deaths due to mesothelioma, with an expected 65 to 70 more deaths by 2020 (Berry et al., 2012).

The primary cause of mesothelioma is through inhalation of asbestos fibres, with 70 - 80% of patients having a documented exposure to asbestos (Carbone et al., 2002, Chen and Pace, 2012). Whilst murine studies have shown that all asbestos fibres have the capacity to cause mesothelioma when injected, crocidolite is regarded as the most carcinogenic. This is believed to be due to the physical properties of the fibres which allow them to easily enter the lung and cross over into the pleural cavity (Pott et al., 1987, Sebastien et al., 1980).

Following inhalation of asbestos dust, fibres lodge in the pleural cavity. Whilst the generation of DNA mutations is known to be the cause of mesothelioma, the precise method by which this occurs is still unknown. There are a number of observed effects that could account for this. Phagocytosis of asbestos fibres by mesothelial cells can lead

to direct DNA damage or damage to spindle fibres during mitosis (Ault et al., 1995, Hesterberg et al., 1986). Irritation generated by the fibres leads to fibrosis and plaque formation (Chen and Pace, 2012) as well as the recruitment of inflammatory cells such as macrophages (Choe et al., 1997, Mossman and Churg, 1998, Tanaka et al., 1998). These macrophages then attempt, but fail, to clear the fibres, which leads to chronic production of reactive oxygen species (ROS) and induces oxidative DNA damage (Hansen and Mossman, 1987, Shukla et al., 2003, Waris and Ahsan, 2006).

The tumour develops by growing around the outer surface of the pleura before developing into a large solid tumour mass, invading the lung cavity. Due to an accumulation of fluid (pleural effusion) between the visceral and parietal layers of the pleura, patients tend to present with dyspnea (breathing difficulty) or chest pain. Other symptoms include weight loss and fatigue. As the disease progresses, patients experience increased fatigue and dyspnea, as well as continual pain due to the increased tumour burden (Ribak et al., 1988, Yates et al., 1997, Robinson and Lake, 2005).

Diagnosis of mesothelioma is initially performed using a chest x-ray to identify pleural effusion or pleural thickening. This is followed up by further imaging with CT scan and cytology and histology on pleural fluid and biopsies. Histologically the tumours are identified as epithelial, sarcomatous or biphasic, with further classing by subtype (Table 1.5). Diagnosis of subtype is carried out to aid in prognosis.

Table 1.5: Histological subtypes of Mesothelioma

Type	Subtypes
Epithelial	Tubulopapillary, Micropapillary, Trabecular, Acinar, Adenomatoid, Solid, Clear cell, Deciduoid, Adenoid cystic, Signet ring cell, Small cell, Rhabdoid, Pleomorphic
Sarcomatous	Conventional, Desmoplastic, Heterologically differentiated, Lymphohistiocytoid
Biphasic	-

Mesothelioma is inevitably fatal, with current therapies (surgery, radiotherapy and chemotherapy) unable to offer a cure. Procedures such as the removal of pleural fluid and talc pleurodesis offer short-term palliative results. Surgical techniques include pleurectomy/decortification and extrapleural pneumonectomy (EPP). Both techniques are non-complete, as remaining microscopic disease usually reforms locally or distally. Radiotherapy is combined with other therapies such as surgery and can be either prophylactic at the incision site to stop reseeding of the cancer, palliative to defined symptomatic sites, or adjuvant (entire hemithorax) after EPP. The benefit from either technique is poorly defined. Chemotherapy can be either offered after surgery or as a stand-alone treatment. The current front-line chemotherapy regimen is the combination of cisplatin and pemetrexed. A randomized trial of this combination therapy versus cisplatin alone saw an increase in median survival from 9.3 months (cisplatin alone) to 12.1 months (combination) (Vogelzang et al., 2003). There have been no further advances in palliative chemotherapy since these data.

As current therapies are not curative, it is imperative to develop a new therapy. One area to pursue is immunotherapy however; this requires a greater understanding of the effect of mesothelioma on the immune system.

1.4.4.1 The effect of mesothelioma on the immune system

Several studies have investigated the effect of mesothelioma on the immune system. An early study by Lew et al (1986), reported a significant decrease in both total T cell numbers as well as T_{helper} cells in mesothelioma patients. Whilst no increase in the number of $T_{suppresor}$ cells was observed the ratio of $T_{suppressor}$ to T_{helper} was increased. In addition, 70% of mesothelioma patients were shown to have a significant decrease in circulating NK cells. Interestingly, Kubota et al (1985) observed that asbestosis patients lacking any tumours had normal levels of both NK cells and lymphokine-activated killer (LAK) cells (a more potent tumourcidal effector cell). It is possible that the decrease seen in mesothelioma patients could be due to trafficking of these cells to the tumour site. Murine studies have shown an increase of NK cells in both subcutaneous and interperitoneal tumours (Jackaman et al., 2012a, Odaka et al., 2002). The study by

Jackaman et al (2012a), further observed that a lack of NK cells (either by antibody depletion or by the use of knockout mice) induced an increase in the rate of tumour progression. To further complicate matters, the tumour environment in mesothelioma patients also contains asbestos fibres (which are lacking in most murine models). Several studies have shown that asbestos fibres can depress the tumouricidal activity of both NK cells and LAK cells (Haslam et al., 1978, Manning et al., 1991a, Robinson, 1989).

The role of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs in mesothelioma is controversial with some studies reporting that T_{regs} do not increase in mesothelioma patient blood (Meloni et al., 2006). DeLong et al (2005) observed a significantly lower number of T_{regs} in the pleural fluid of mesothelioma patients, as opposed to lung cancer patients. They hypothesized that mesothelioma is a more localized cancer; with increased suppressive cells most likely occurring within the tumour, rather than systemically. Indeed, histological studies of human mesothelioma tumours have shown a large infiltration of T_{regs} within the tumour microenvironment (Hegmans et al., 2006), supporting this theory. Hegmans et al (2006) further characterized the infiltration of other immune cells into human mesothelioma tumours and showed they consist of T cells and macrophages with minimal infiltration of DCs (Langerhan, myeloid and plasmacytoid). It is possible that the immune environment within and around the tumour impairs DC infiltration, maturation and function. The analysis of cytokines present in the pleural effusions of mesothelioma patients identified high concentrations of TGFβ, VEGF and IL-6 (DeLong et al., 2005), which as previously mentioned impairs the maturation of DCs.

Functionally, there are very few studies showing the effect of mesothelioma on the immune cells of patients. Bromelow et al (2001) observed that peripheral blood lymphocytes from mesothelioma patients had no impairment in their ex-vivo proliferative capacity by mixed lymphocyte reaction compared with healthy age matched controls. A study by Ho et al (2005) observed that antibodies specific to mesothelin (a protein expressed on mesothelial cells and over-expressed on mesothelioma cells) was detectable in the sera of mesothelioma patients. This indicates that the humoral arm of the immune system appears to remain functioning.

Murine studies have attempted to further characterize the effect of mesothelioma on the function of immune cells. In contrast to the previously mentioned study by Hegmans et al (2006), murine studies have shown the infiltration of DCs into the tumour microenvironment (Jackaman et al., 2009). Several studies have shown the capacity for DCs to present tumour antigen in a murine mesothelioma model. Marzo et al (1999) used a HA (influenza hemagglutinin) transfected mesothelioma model to show the generation of a tumour specific $CD8^+$ T cell proliferation in the draining lymph node of tumour bearing mice. Similarly, the use of an OVA-transfected mesothelioma model also generated a tumour-specific CTL response (Jackaman et al., 2012b, Jackaman et al., 2003, Jackaman et al., 2009). Neither model was able to generate tumour rejection, indicating impairments in the pathway. Failure in the HA-model was determined to be due to a lack of migration of generated $CD8^+$ T cells back to the tumour site. Rather these cells disappeared over several weeks. Further studies have shown a requirement of $CD4^+$ T cells for both $CD8^+$ T cell survival as well as the ability to migrate back to the tumour site (Marzo et al., 2000). $CD8^+$ T cells generated in the OVA-transfected model were shown to traffic back and accumulate in the tumour site. Whilst these cells were shown to be activated as noted by high expression of CD25 and CD44, their CTL activity was suppressed. $CD4^+$ T cells were also observed in the tumour environment and were shown to be effector rather than regulatory. In contrast to the human studies by Hegmans et al (2006), T_{reg} cells had a minimum involvement. No increase of T_{regs} was observed in the draining lymph nodes and systemic depletion of T_{regs} did not stop the suppression of CTL activity in the tumours (Jackaman et al., 2009). The difference in response between the two murine models has been associated with the neoantigen used. The HA antigen is membrane bound therefore requiring processing and transport to the lymph nodes by tumour APCs, whilst the OVA antigen is secreted, allowing processing to occur in the lymph node itself by lymph node resident DCs. In a study by Nelson et al (2001), the lack of an anti-tumour response being generated in these models was hypothesised to be due to effects of the tumour microenvironment. Therefore suppressive factors generated by the tumour microenvironment could be affecting different populations of immune cells (DCs in the HA model, CTLs in the OVA model).

In a recent study by Jackaman et al (2013) tumour supernatant from a murine mesothelioma model polarised macrophages to a M2 phenotype indicating one potential mechanism by which the tumour develops a suppressive microenvironment.

1.5 Immunotherapy

As many cancers show limited improvement in survival following standard therapies, there is a growing interest in the area of immunotherapy. Immunotherapy involves the administration of compounds which activate, modulate or switch on/off specific immune responses. These include cytokine therapies, TLR agonists, blocking antibodies and adoptive cell therapies (Khong et al., 2012, Devaud et al., 2013). Whilst development of successful immunotherapies has been slow, fourteen monoclonal antibody therapies have been approved by the FDA to-date (Table 1.6). Promising results in animal models and human clinical trials has identified agonist αCD40 as a potentially successful monoclonal antibody therapy for mesothelioma (Jackaman et al., 2011, Jackaman et al., 2012a, Jackaman et al., 2008, Jackaman and Nelson, 2012).

Table 1.6: Antibody therapies and their targets (adapted from Sliwkowski and Mellman (2013))

Drug name	Target	Function	Cancer
Cetuximab	EGFR	Inhibits cell division, activates compliment, triggers antibody dependent cellular cytotoxicity	Colon, head and neck
Panitumumab	EGFR	Inhibits cell division and activates compliment	Colon
Trastuzumab	HER2	Triggers antibody dependent cellular cytotoxicity	Breast, gastric
Pertuzumab	HER2	Inhibits dimerization of HER2 leading to a decrease in tumour growth rate	Breast
Ado-trastuzumab emtansine	HER2	Binds to HER2 and releases toxin – killing cancer cell	Breast

Bevacizumab	VEGF	Binds to VEGF inhibiting its function in angiogenesis	Colon, NSCLC, glioblastoma, kidney
Ipilimumab	CTLA-4	Blocks CTLA-4 inhibitory signal	Melanoma
Rituximab	CD20	Activates compliment and triggers antibody dependent cellular cytotoxicity	Lymphoma, chronic lymphocytic leukemia
Ofatumubab	CD20	Activates compliment	chronic lymphocytic leukemia
^{90}Y-labeled ibritumomab tiuxetan	CD20	Activates compliment, triggers antibody dependent cellular cytotoxicity and kills cell through attached radioisotope	Lymphoma
^{131}I-labeled tositumomab	CD20	Kills cell through attached radioisotope	Low grade non-Hodgkin's lymphoma
Brentuximab vedotin	CD30	Kills cell through attached cytotoxic drug	Hodgkin's lymphoma
Gemtuzumab ozogamicin	CD33	Kills cell through attached cytotoxic drug	Acute myelogenous leukemia
Alemtuzumab	CD52	Triggers antibody dependent cellular cytotoxicity	Chronic lymphocytic leukemia
Nivolumab	PD-1	Blocks PD-1 ligand inhibitory signal	Lung cancer, melanoma, renal carcinoma

1.5.1 CD40 and its ligand

CD40 was first identified on B cells and described as a B cell growth activator (Clark and Ledbetter, 1986, Paulie et al., 1985). It is a 48 kDa transmembrane glycoprotein surface receptor and a member of the Tumour Necrosis Factor Receptor superfamily (TNFRSF) (Banchereau et al., 1994). Apart from B cells, it is also expressed on other APCs (DCs and macrophages), non-immune cells (such as epithelial cells, endothelial cells and haemopoetic progenitors) as well as activated T cells and some tumour cells (Grewal and Flavell, 1996, Stout and Suttles, 1996, Van Kooten and Banchereau, 1996).

The ligand for CD40, CD154 (also known as TRAP, T-BAM, Bp35 or CD40L) is a 34–39 kDa type II integral membrane protein (Quezada et al., 2004). It is expressed on a wide variety of cells including activated T cells (Klaus et al., 1997), monocytes (Filion et al., 2003), endothelial cells (Mach et al., 1997), platelets (Andre et al., 2002), basophils, eosinophils and activated human DCs (Grewal and Flavell, 1998). Studies have shown that on human blood DCs the expression of CD40 (Hellman and Eriksson, 2007) and its ligand CD40L (Pinchuk et al., 1996), whilst being minimally expressed at resting state, are increased significantly following stimulation.

1.5.2 CD40-CD40L

The interaction of CD40 with CD40L, or with agonist anti-CD40 antibodies, induces a number of effects depending on the target cell involved. Activating $CD40^+$ tumour cells has been shown to directly inhibit the growth of human breast cancer cells or induce apoptosis in ovarian cancer cells (Eliopoulos et al., 2000, Tong et al., 2001). Activating tumour endothelial cells through surface CD40 leads to blood vessel activation which becomes permissive to immune cell traffic (Hamzah et al., 2008). Activating B cells through surface CD40 leads to clonal expansion, the generation of plasma cells and isotype switching (Berberich et al., 1994, Kawabe et al., 1994, Xu et al., 1994).

Activating DCs through surface CD40 induces their maturation, including upregulation of co-stimulatory and MHC molecules, IL-12 production and an increased capacity to induce T cell proliferation (Caux et al., 1994, Cella et al., 1996, Koch et al., 1996, Peguet-Navarro et al., 1995). CTL-priming to achieve an anti-tumour response is dependent on $CD4^+$ T cell help. Studies have shown that in the lack of $CD4^+$ T cell help, activation of CD40 signalling in DCs by agonist αCD40 can replace $CD4^+$ T cell help and achieve an anti-tumour CTL response (Bennett et al., 1998, Fransen et al., 2011, Schoenberger et al., 1998, Toes et al., 1998). These roles highlight the potential for activating immune cells and non-immune cells via CD40 as an immunotherapy.

1.5.3 CD40-based immunotherapy for mesothelioma

There have been several studies investigating the potential of CD40-based therapy in murine mesothelioma models. Friedlander et al (2003) showed that whilst gene delivery of CD40L via and adenoviral vector had no effect in vitro, there was an effect in vivo. Ex-vivo treatment of a murine mesothelioma tumour cells with the CD40L adenoviral vector resulted in failure of subsequent in-vivo tumour growth. In-vivo intra-tumoural injection of the plasmid induced regression of treated site and untreated distal tumours, and generated tumour specific $CD8^+$ T cells, which conferred protection when adoptively transferred into naïve mice. Jackaman et al (2008, 2012, 2011) showed that an agonist αCD40 antibody could induce the complete regression of small mesothelioma tumours. When agonist αCD40 antibody was combined with IL-2 large tumours regressed. Whilst depletion of $CD4^+$ T cells had no effect on CD40-induced tumour regression, a loss of response was observed when $CD8^+$ T cells or B cells were depleted. A similar requirement for $CD8^+$ T cells and independence of $CD4^+$ T cells has been observed in mesothelioma tumours in Balb/c mice (Stumbles et al., 2004). Similar to Friedlander et al (Friedlander et al., 2003), Jackaman et al observed both the generation of memory and the reduction of distal tumours following intra-tumoural αCD40 treatment of a single tumour. The authors showed a requirement of $CD4^+$ T cells, $CD8^+$ T cell and NK cells for the resolution of untreated distal tumours (Jackaman et al., 2012a, Jackaman and Nelson, 2012).

As previously mentioned, current treatment for mesothelioma involves surgery and/or chemotherapy. A study by Nowak et al (2003) showed that synergy could be achieved by treating mice with mesothelioma tumours firstly with Gemcitabine then followed with αCD40. Depletion studies identified that whilst CD4 cells were not required, CD8 cells were critical for the anti-tumour response. Broomfield et al (2005) and Khong et al (2013) subsequently investigated agonist αCD40 immunotherapy in combination with either surgery and / or chemotherapy in mice. Treatment with αCD40 following debulking (removal of 75% of tumour mass) delay tumour growth, but did not improve overall survival. Following treatment with gemcitabine alone, there was an increase in survival and the development of memory responses. The inclusion of αCD40

immunotherapy following chemotherapy increased the number of mice cured and the percentage of mice that developed protective memory. When the tumour mass was completely removed, αCD40 offered no benefit over chemotherapy alone, and no memory was developed (Broomfield et al., 2005). Khong et al (2013) attempted to simulate the microscopic presence of tumour cells after surgery by injecting fresh tumour cells following complete resection of tumour mass. In this study, treatment with αCD40 led to a significant slowing of tumour growth and increased survival and slowed the development of distal tumours.

The results from the study by Nowak et al (Nowak et al., 2003) has led to beginning of human trials of αCD40 immunotherapy combined with chemotherapy. A phase 1b study combining αCD40 with cisplatin and pemetrexed has been completed and collected samples are currently being analysed for various immunological responses (Khong et al., 2012).

The most recent study by Khong et al (2014) investigated agents that targeted DCs in the aim to activate them and generate a CTL response against murine mesothelioma. They observed increased CTL activity and improved survival following administration of either the TLR7 agonist imiquimod or agonist anti-CD40 post debulking surgery. Indeed, the combination of imiquimod and anti-CD40 further improved survival. Whilst not measuring specific activation of DCs, they hypothesized that DC activation by these agents could explain the improved response. Therefore futher studies are required to determine whether CD40 stimulation of mesothelioma patient DCs leads to their activation and potential to generate CTLs. Possible opening a pathway for therapies which could improve the outcome for patients.

1.6 Aims

Dendritic cells have an important role in establishing and directing immune responses. Whilst studies show that DCs are altered by both age and the presence of cancer, the published data is usually highly variable. With current therapies for malignant mesothelioma doing little to halt its aggressive nature and poor outcome, new therapies; such as immunotherapies, are being thoroughly investigated. As little is known on how DCs are affected by the presence of mesothelioma and their key role in most immunotherapies, further studies were required to assess this. Therefore, the principle aims of this thesis were:

1. To investigate the effect of aging on dendritic cell subsets in healthy individuals
    a. To determine changes in numbers of DCs in the blood
    b. To determine if monocyte derived DCs from elderly healthy individuals are functionally or phenotypically impaired in their ability to differentiate or mature.

2. To investigate the effect of mesothelioma on dendritic cell subsets.
    a. To determine changes in numbers of DCs in the blood
        i. To evaluate whether enumeration correlates with survival time
    b. To determine if monocyte derived DCs from mesothelioma patients are functionally or phenotypically impaired in their ability to differentiate or mature.
        i. To determine if improved response to stimulation corresponds with an improved survival time

3. To investigate whether impairments due to age or mesothelioma can be recovered thru CD40 licensing
    a. To evaluate whether the response to CD40 activation by MoDCs from people with mesothelioma correlates with survival time.

4. To determine if soluble factors from mesothelioma cell lines impair healthy DC function

5. To investigate if therapy restores DC number and function.

2 MATERIALS AND METHODS

2.1 Human Ethics

Approval for this study was given by the Ethics Committees of the Sir Charles Gairdner Hospital, Perth, Western Australia (#2008-041); the Mount Hospital, Perth, Western Australia (#EC50.1), and the Human Ethics committee at Curtin University of Technology, Bentley, Western Australia (#HR68/2008).

2.2 Recruitment of participants – Patients and Healthy Donors

Healthy volunteers were recruited by (i) radio advertising and interviewing on CurtinFM, (ii) print advertising in an elderly demographic newspaper, (iii) poster advertising at Curtin University and (iv) recruitment from laboratory volunteers. Signed consent was obtained by the PhD student collecting the blood. Health status was determined by collecting data on current and past medical conditions of both the volunteer and biological family (refer to appendix 1). Young volunteers were aged between 20 and 45 years of age. Elderly volunteers were aged between 55 and 85 years of age.

People with mesothelioma were recruited by three clinicians, with three additional people recruited through radio advertising on CurtinFM. Signed consent was obtained by either the recruiting clinician or by the PhD student collecting the blood. Patients were excluded from these studies if they had undergone active anti-cancer treatment (chemotherapy, radtiotherapy or surgery) in the previous 9 months.

2.3 Collection of blood samples

Whole anti-coagulated blood was collected via mid-arm vena-puncture from healthy volunteers and patients into five 10 ml K_2EDTA vacutainer tubes (BD Pharmingen, USA) and transported to the laboratory for processing.

2.4 Cell Culture and Maintenance

a. Tumour cell lines

Two human tumour cell lines were used in this research. The MM cell lines JU77 and ONE58 as described previously (Manning et al., 1991b) were kindly donated by Dr. Simon Fox (School of Pharmacy, Curtin University of Technology, Australia).

All tumour cell lines were maintained in RPMI (Invitrogen, USA) media containing 10 % FCS (Thermo Fisher Scientific, USA), 50 μM 2ME (Sigma-Aldrich, USA), 100 U/ml Benzylpenicillin (Invitrogen, USA) and 50 mg/ml Gentamycin (Invitrogen, USA). Cells were cultured in a 37º C and 5 % CO_2 humidified incubator until 80 % confluent.

All cell lines were adherent and required trypsinising during passage and freezing down. Briefly, once cells obtained 80 % confluency, media was removed and the mono-layer was washed in 10 ml PBS (Invitrogen, USA). Following removal of PBS, 1 ml of trypsin (Sigma-Aldrich, USA) was added and the flask was incubated at 37º C and 5 % CO_2 for 3 min to remove adherent cells. Cells were then resuspended in media and washed by centrifugation at 1200 rpm for 5 min.

b. Thawing of cells

Cells which had been stored in cryovials (2 ml; Corning, USA) at -80º C were thawed rapidly and immediately transferred to a 15 ml tubes containing 9 ml of cultrure media. Cells were then centrifuged at 1200 rpm for 5 minutes. The media was then removed and the cells resuspended in 15 ml of media and aliquoted into a 75 cm^2 flask for culturing.

c. Cell viability and counting

Cell viability and counting was performed by trypan blue staining. Briefly, 10 μl of cells was gently mixed with 10 μl of 0.4 % trypan blue solution (Sigma-Aldrich, USA). Following which, 10 μl of the mixture was loaded onto a haemocytometer and viable (unstained) and dead (blue stained) cells were counted.

d. Cryopreservation of cells

All cell lines were frozen down in a freezing mixture of 60 % culture media, 20 % FCS and 20 % dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). Trypsinised cells were washed (1200 rpm for 5 minutes) in cell culture media and then counted. Cells were then centrifuged and resuspended in freezing mixture at a concentration of 1 x 10^6 cells/ml. Cells were then aliquoted into cryovials at 1 ml/vial and stored at -80º C.

e. Collecting tumour supernatants

Once confluency of tumour cells was determined to be 80 %, the culture media was removed and cells were washed in 5 ml of PBS. Following washing, fresh culture media was added to the flasks and cells were allowed to grow for a further 24 hours. After 24 hours, the supernatant was collected into a 50 ml tube and centrifuged at 1200 rpm for 5 minutes to pellet any cell debris. The debris free supernatant was then collected into a fresh tube and stored at -80º C until needed.

2.5 Enumeration of pDCs, mDC1s and mDC2s from whole blood

Enumeration of three blood DC subsets was measured using a Blood Dendritic Cell Enumeration Kit (Miltenyi-Biotec, Germany). Briefly, 300 µl of whole blood was aliquoted into two 5 ml polystyrene round-bottom tubes labeled 'test' or 'control'. To the 'test' tube, 10 µl of an anti-BDCA cocktail (containing BDCA1-PE (clone AD5-8E7), BDCA2-FITC (clone AC144), BDCA3-APC (clone AD5-14H12), CD19-PeCy5 and CD14-PeCy5) and 5 µl of dead cell discriminator (a fluorescent photolytic dye) was added. To the 'control' tube, 10 µl of the control cocktail (containing Mouse IgG2a-PE, Mouse IgG1-FITC, Mouse IgG1-APC, CD19-PeCy5 and CD14-PeCy5) and 5 µl of dead cell discriminator was added. The tubes were then incubated horizontal for 10 min on ice under a 60W globe. Following incubation, 4 ml of 1x red blood cell lysis buffer (1.55 M NH_4CL, 0.1 M $KHCO_3$, 1 mM EDTA, ph 7.4) was added to each tube and the tubes further incubated at room temperature for 10 min in the dark. Tubes were then centrifuged at 300 *g* for 5 min, the supernatant discarded and the pellet washed twice in 4 ml of FACS Buffer (1x PBS containing 1 % BSA (Sigma-Aldrich, USA), 2 % FCS and 0.01 % sodium azide). Following the washes the supernatant was discarded and the

pellet resuspended in 300 µl of FACS buffer, 150 µl of Fix Solution (3.7 % formaldehyde in PBS) and 5 µl Discriminator Stop Reagent (Deoxyribonucleic acid in 10 mM TRIS, 10 mM NaCL, 1 mM EDTA, pH8). Samples were analysed by cytometry on a FACSCantoII (BD Biosciences) using Diva software.

2.6 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMC) were obtained from blood samples by Ficoll-paque density gradient centrifugation. Briefly, for collection volumes less than 20 ml the blood was aliquoted into one 50 ml tube. For collection volumes between 20 and 45 ml the blood was aliquoted evenly into two 50 ml tubes. For collection volumes greater than 45 ml, blood was aliquoted into three 50 ml tubes. To each tube PBS containing 2 mM EDTA (Sigma-Aldrich, USA) was added to a total volume of 35 ml per tube. The 35 ml of diluted blood was then layered over 15 ml of Ficoll-paque PLUS (GE Healthcare, Sweden) and centrifuged at 400 *g* for 40 min at 20º C with the brake off. The interphase (containing lymphocytes, monocytes and thrombocytes) was carefully removed and resuspended in 50 ml of PBS/2 mM EDTA. The tubes were then centrifuged at 300 *g* for 10 min and the supernatant discarded. The cells were then resuspended in 50 ml of PBS/2 mM EDTA and centrifuged at 200 g for 10 min at 20º C twice to remove platelets.

2.7 In-vitro generation of MoDCs

Dendritic cells were prepared using a modified procedure to that used by Romani et al (Romani et al., 1994). Briefly, PBMC (1×10^8 cells) were allowed to adhere to a 75 cm^2 tissue culture flask in RPMI media containing 10 % FCS, 50 µM 2ME, 100 U/ml Benzylpenicillin and 50 mg/ml Gentamycin. Following a two hour incubation at 37º C, 5 % CO_2, non-adherent cells were removed and the subsequent adherent cells (monocytes) cultured for 7 days in 80 ng/ml GM-CSF (Shenandoah, USA) and 10 ng/ml IL-4 (R&D Systems, USA) added on days 0 and supplemented on day 4. The MoDCs cultures continuously contained 10 µg/ml Polymixin-B (Sigma-Aldrich, USA) to inactivate lipopolysacharides (LPS).

2.8 Isolation of lymphocytes

Peripheral blood mononuclear cells (1 x 10^8 cells) were allowed to adhere to a 75 cm^2 tissue culture flask in RPMI media containing 10 % FCS, 50 μM 2ME, 100 U/ml Benzylpenicillin and 50 mg/ml Gentamycin. Following a two hour incubation at 37º C, 5 % CO_2, non-adherant cells were collected containing a predominantly lymphocyte population.

2.9 Stimulation of MoDCs with cytokines

On day 7 of in-vitro generation of MoDCs, non-adherent cells (comprised of immature MoDCs) were collected into a 50 ml tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in 50 ml of PBS and centrifuged at 1200 rpm for 5. Following centrifugation the cells were resuspended in 10 ml of culture media and 2 ml was aliquoted in to five 75 cm^2 flasks, which was then made up to 15 ml with culture media. To each flask 80 ng/ml GM-CSF and 10 ng/ml IL-4 was added. MoDCs were stimulated for 2 days with either 10 μg/ml LPS (Sigma-Aldrich, USA), 20 ng/ml IFNγ (Sigma-Aldrich, USA), 10 μg/ml LPS and 20 ng/ml IFNγ or 0.66 μg/ml CD40L (Genscript, USA). One flask was used as a no-stimuli control containing 10 μg/ml Polymixin-B.

2.10 Phenotyping of MoDCs

Following 48 hr stimulation, non-adherent cells and weakly-adherent cells were collected in a 15 ml tube. The tubes were centrifuged at 1200 rpm for 5 minutes. Two ml of supernatant was then aliquoted into cryovials and stored at -80º C for cytokine analysis. Stimulated and unstimulated cells were stained for cell surface expression of CD1a-PeCy5 (clone HI149), CD11c-APC (clone B-ly6), CD14-FITC (clone M5E2), CD40-PeCy5 (clone 5C3), CD80-PE (clone 2D10), CD83-APC (clone HB15E), CD86-PE (clone 2331 (FUN-1)) and HLA-DR-APC-Cy7 (clone L242) using directly conjugated antibodies (BD Pharmingen, USA). Cells were incubated for 30 min at 4º C in the dark then washed in PBS. Cells surface expression was measured by flow

cytometry. As multicolour immunofluorescence (see Appendix 9.3 for panels) was used for the analysis, unstained, isotypes and single stained samples were used as controls. Due to the low number of cells present in patient samples, fluorescence minus one (FMO) controls could not be used, background fluorescence was determined by isotype and unstained controls.

2.11 Measurement of antigen processing capacity

Immature MoDCs (5 x 10^4 cells) were resuspended in RPMI media containing 10 % FCS, 50 μM 2ME, 100 U/ml Benzylpenicillin and 50 mg/ml Gentamycin. Cells were incubated for 1 hr at 37º C with or without 10 μg/ml DQ-OVA (Invitrogen, USA). Cells were also incubated at 4º C with or without DQ-OVA as a control. Antigen processing capacity was determined by measurement of FITC emission by flow cytometry and calculated using the formula in Appendix 9.2.

2.12 CFSE labelling of T-cells

PBMCs from different donors were isolated as above. Following incubation of PBMCs for two hours, the non-adherent population (containing monocyte-depleted, T cell enriched cells) was collected, washed in PBS and resuspended at 2 x 10^7 cells/ml in RPMI media containing 3.5 μM CFSE (Sigma-Aldrich, USA). Cells were incubated at room temperature for 10 min, then washed three times with RPMI media and used as responder cells for the mixed lymphocyte reaction (MLR).

2.13 Mixed Lymphocyte reaction

Stimulated MoDCs were seeded in duplicate into a 96 well plate at concentrations ranging from 1 x 10^3 to 1 x 10^5 cells/ml. To each well, 2 x 10^5 CFSE-labelled T cells were added. Control wells contained MoDCs alone, T cells alone and T cells stimulated with Concanavalin A (Sigma-Aldrich, USA). Plates were incubated in the dark at 37º C 5% CO_2 for 8 days. Following incubation, the cells were washed in PBS and stained for expression of CD4-PE (clone RPA-T4) and CD8-APC (clone RPA-T8) using directly conjugated antibodies (BD Pharmingen, USA). Cells were incubated for 30 min at 4º C

in the dark. Cells were further washed in PBS to remove excess antibody and analysed by flow cytometry for CD4 and CD8 proliferation.

2.14 Measurement of cytokine secretion by Cytokine Bead Array

Supernatatants from MoDC cultures stimulated for 48 hrs with 10 µg/ml LPS were collected and stored at -80º C until analysed. Cytokines TNFα, IL-10, IL-12(p70), VEGF and IFNγ were measured simultaneously by cytometric bead array (BD Pharmingen, USA) as per the manufacturer's protocol. Briefly, a single vial of a known standard for each cytokine was pooled and reconstituted in 4 ml of Assay Diluent. This was then serially diluted (1:2) to a final dilution of 1:256. Frozen supernatants were allowed to thaw on ice. The assay was performed in a 96 well plate with 5 µl of diluted Capture Beads added to each assay well. To each assay well, 5 µl of standard or neat sample was added and mixed gently. Following incubation of the plate at RT for 1 hour, 5 µl of the mixed PE Detection Reagent was added to each assay well. The samples were mixed gently then the plate was incubated at RT for 2 hours. Following incubation the assay wells were washed one time in Wash Buffer. Beads were resuspended in 100 µl of wash buffer then analysed on the FacsCantoII.

2.15 Co-culturing of MoDCs with tumour supernatant

Immature MoDCs were incubated for 48 hours in either 100 % culture media or culture media containing 50 % media and 50 % tumour supernatant (from either JU77 or ONE58 cells). During the 48 hour incubation the immature MoDCs were stimulated with either LPS or three different concentration of CD40L (1x: 0.66 µg/ml, 5x: 3.3 µg/ml, 25x: 16.5 µg/ml). Following incubation, cells were collected and analysed for changes in phenotype as previously described (Chapter 2.10)

2.16 Statistics

Statistical analysis was conducted using GraphPad Prism v4.03 (USA). Data was expressed as mean ± standard error of the mean (SEM) and where relevant a line of linear regression was plotted. Statistical difference was determined by a two-tailed

Mann-Whitney test or an unpaired T test. P-values less than 0.05 were considered statistically significant.

3 DENDRITIC CELLS AND THE EFFECT OF AGE

3.1 Introduction

Immunosenescence is characterised by a steady decrease in the ability of the immune system to respond to infection and vaccines during aging, and is believed to be the underlying reason for the increased susceptibility to cancer seen in the elderly (Larbi et al., 2008, Pawelec and Larbi, 2008). Responses to specific antigens decline, with defects in humoral and T cell responses to antigen in aged individuals reported (Weiskopf et al., 2009). T cells undergo a shift from the naive to the memory phenotype and demonstrate reduced proliferative responses and impaired cytolytic activity (Fahey et al., 2000, Rukavina et al., 1998, Walford et al., 1981). The production of high-affinity antibodies by B cells also decreases in aging hosts (Dunn-Walters and Ademokun, 2010, Frasca et al., 2011).

Dendritic cells (DC), key antigen presenting cells, link and regulate the innate and adaptive immune systems (Banchereau et al., 2000, Banchereau and Steinman, 1998). Dendritic cells derive from bone marrow progenitors and reside in peripheral tissues or travel in circulation as immature phagocytic cells. Immature dendritic cells capture antigens in tissue sites, then during maturation, process and transport these antigens to lymph nodes for T cell priming. Dendritic cells rapidly differentiate and mature in response to various stimuli to produce pro- or anti-inflammatory cytokines that influence the outcome of the immune response.

Dendritic cells are divided into distinct subsets that differ in phenotypes, tissue distribution and function (Heath et al., 2004, Villadangos and Schnorrer, 2007). Circulating or Blood DCs, comprise of both myeloid and plasmacytoid DCs. The myeloid or conventional DCs (mDC) are made up of at least two subsets and have been shown to originate from either $CD34^+$ progenitors or $CD14^+$ monocytes (Caux et al., 1997, Caux et al., 1996, Romani et al., 1994). The more common mDC-1 cell type is a major stimulator of T cells, it has been shown to secrete IL-12 to induce polarization of naive $CD4^+$ T cells into Th1 cells (Rissoan et al., 1999). The rarely occurring mDC-2

cell type may play a role in cross presentation as they share many genetic similarities to the $CD8^+$ DC in mice (Robbins et al., 2008). However, both mDC subpopulations are efficient in uptake, processing, and presentation of foreign antigens. Plasmacytoid DCs (pDCs) are generated from either $CD34^+$ progenitors or $CD11c^-$ blood precursors and rapidly secrete large amounts IFN-α in response to microbial challenge, playing a key role in anti-viral immunity (Blom et al., 2000, Grouard et al., 1997, Siegal et al., 1999, Soumelis and Liu, 2006). Due to the low numbers of blood DCs, functional studies are often conducted using monocyte derived dendritic cells (MoDCs). First demonstrated by Sallusto and Lanzavecchia (1994), monocytes can be differentiated in vitro into DCs by culturing with GM-CSF and IL-4. In vivo differentiation was later observed by Randolph et al (1999) in mice. It has been hypothesized that MoDCs represent a population of APCs that can be generated rapidly due to an inflammatory response where the circulating blood DCs are inadequate (Hart, 1997).

There are conflicting results in regard to whether DC numbers, function or phenotype are altered with age. This may be due to the heterogeneous nature of dendritic cells, with differential effects on different subsets. Most studies looking at the effect of aging on circulating DC subsets in humans identified a significant decrease in pDC numbers with no change in mDC numbers (Shodell and Siegal, 2002, Perez-Cabezas et al., 2007, Orsini et al., 2012, Jing et al., 2009). In contrast, Della-Bella et al (2007) identified a decrease in mDC populations with no change in pDC population numbers.

Due to the low numbers of circulating DCs, functional studies were conducted using MoDCs. Whilst the majority of studies (Steger et al., 1996, Lung et al., 2000, Ciaramella et al., 2011, Agrawal et al., 2007) show that cell surface markers for iMoDCs do not significantly vary due to age, Pietschmann et al (2000) observed a significant drop in HLA-DR expression on iMoDCs derived from elderly subjects. A few studies have investigated age-related phenotypical changes of MoDCs following stimulation. No differences were observed following stimulation with LPS (Agrawal et al., 2007, Ciaramella et al., 2011) or with influenza virus (Lung et al., 2000, Saurwein-Teissl et al., 1998).

The primary role of DCs is to take up and process antigen for presentation to T cells. To date, only one study (Agrawal et al., 2007) investigated whether antigen uptake is affected by aging. They observed that increasing age was associated with significantly decreased antigen uptake irrespective of the mechanism involved, i.e. mannose-receptor mediated, macropinocytosis or phagocytosis. Until now, no study had investigated whether antigen processing is affected by age.

Several studies have investigated whether antigen presentation is affected by age. Steger et al (1996, 1997) observed that DCs pulsed with tetanus toxoid (TT) from elderly or young individuals induced similar levels of proliferation of TT-specific T cells. Lung et al (2000) observed similar results with influenza-stimulated DCs. No study to-date had systematically examined DC responses to stimulation using IFNγ with or without LPS.

The studies presented in this chapter aimed to assess the numbers of pDCs, mDC-1 and mDC-2 cells in human peripheral blood across the spectrum of aging, from healthy young adults, starting at 21 years old, up to and including healthy elderly volunteers of 85 years of age. The antigen processing and presentation functions of GM-CSF/IL-4 monocyte MoDCs were assessed along with their capacity to respond to microbial and cytokine maturation stimuli.

3.2 Results

3.2.1 Characteristics of study volunteers

A total of 67 volunteers were recruited for this study by (i) radio advertising, (ii) print advertising in an elderly demographic newspaper, (iii) poster advertising at Curtin University, and (iv) recruitment from laboratory volunteers. Signed consent was obtained immediately prior to blood collection. Health status was determined by collecting data on current and past medical conditions of the volunteer and their biological family using a survey form. Volunteers were excluded from the study if they currently had or were being treated for cancer, autoimmune disorders or other severe immune disorders. For the purposes of this study, ages were dichotomised into two groups: 'young' volunteers were aged between 20 and 44 years of age whilst 'elderly' volunteers were aged between 56 and 84 years of age (Table 3.1). Compiled data (Appendix 1) indicated that young volunteers had significantly less past medical conditions ($p = 0.006$), with significantly more elderly volunteers suffering from blood or cholesterol disorders ($p = 0.007$). Significantly more young volunteers believed that they had not been exposed to asbestos throughout their lifetime ($p < 0.0001$). Approval for this study was given by the Human Ethics Committees for the Sir Charles Gairdner Hospital, Perth, Western Australia (#2008-041); the Mount Hospital, Perth, Western Australia (#EC50.1), and for Curtin University, Bentley, Western Australia (#HR68/2008).

Table 3.1: Characteristics of study volunteers

	Young	**Elderly**	**p Value**
Number of subjects:	30	37	
Age range in years:	20 – 44	56 - 84	
Mean age in years:	28.77	68.46	
Standard deviation of age:	7.793	6.371	
Female gender no. (%):	21 (70)	14 (38)	0.0225

3.2.2 Human plasmacytoid dendritic cell numbers decrease with age

Blood from 54 of the 67 healthy volunteers with ages ranging from 21 to 84 years of age (Table 3.1) was used to investigate whether there were any changes to blood dendritic cell numbers during aging. Cell numbers per ml of blood were determined using a blood dendritic cell enumeration kit and flow cytometry. Gates were set to eliminate debris, red blood cells, B-cells, monocytes and granulocytes (Figures 3.1a and 3.1b). The remaining cells contain the sub-populations of pDCs, mDC1s and mDC2s, which were identified by high expression of BDCA-2 (CD303; pDCs), high expression of BDCA-1 (CD1c; mDC1s) (Figure 3.1c) and very high expression of BDCA-3 (CD141; mDC2s) (Figure 3.1d).

No age-related changes were observed for mDC1s (Figure 3.1e; $p = 0.74$) and mDC2s (Figure 3.1f; $p = 0.41$). In contrast, pDC numbers significantly decreased with age (Figure 3.1g; $p = 0.0004$). Note that the statistical test used was linear regression of continuous samples.

3.2.3 Age does not reduce the capacity of monocytes to differentiate into immature MoDCs

As functional analyses of blood dendritic cell subsets are difficult due to low yields an alternative approach is to study dendritic cells generated from monocyte precursors *in vitro*, referred to as monocyte-derived DCs (MoDCs). Thus, monocytes from 45 healthy volunteers were exposed to GM-CSF and IL-4 and investigated for their ability to differentiate into immature (i)MoDCs (Figure 3.2a) by examining surface expression of key DC markers on gated $CD14^-$ cells (Figure 3.2b).

CD11c (Figure 3.2c) is a classical dendritic cell marker as well as a co-stimulatory molecule involved in T-cell to dendritic cell interaction. There was no difference between the percentage of dendritic precursor cells that had differentiated into $CD14^-$ $CD11c^+$ dendritic cells between young versus elderly ($p = 0.51$) volunteers (Figure 3.2d). Whilst there appeared to be a downward trend in CD11c surface expression levels with age, measured by mean fluorescent intensity (MFI; Figure 3.2e), individual

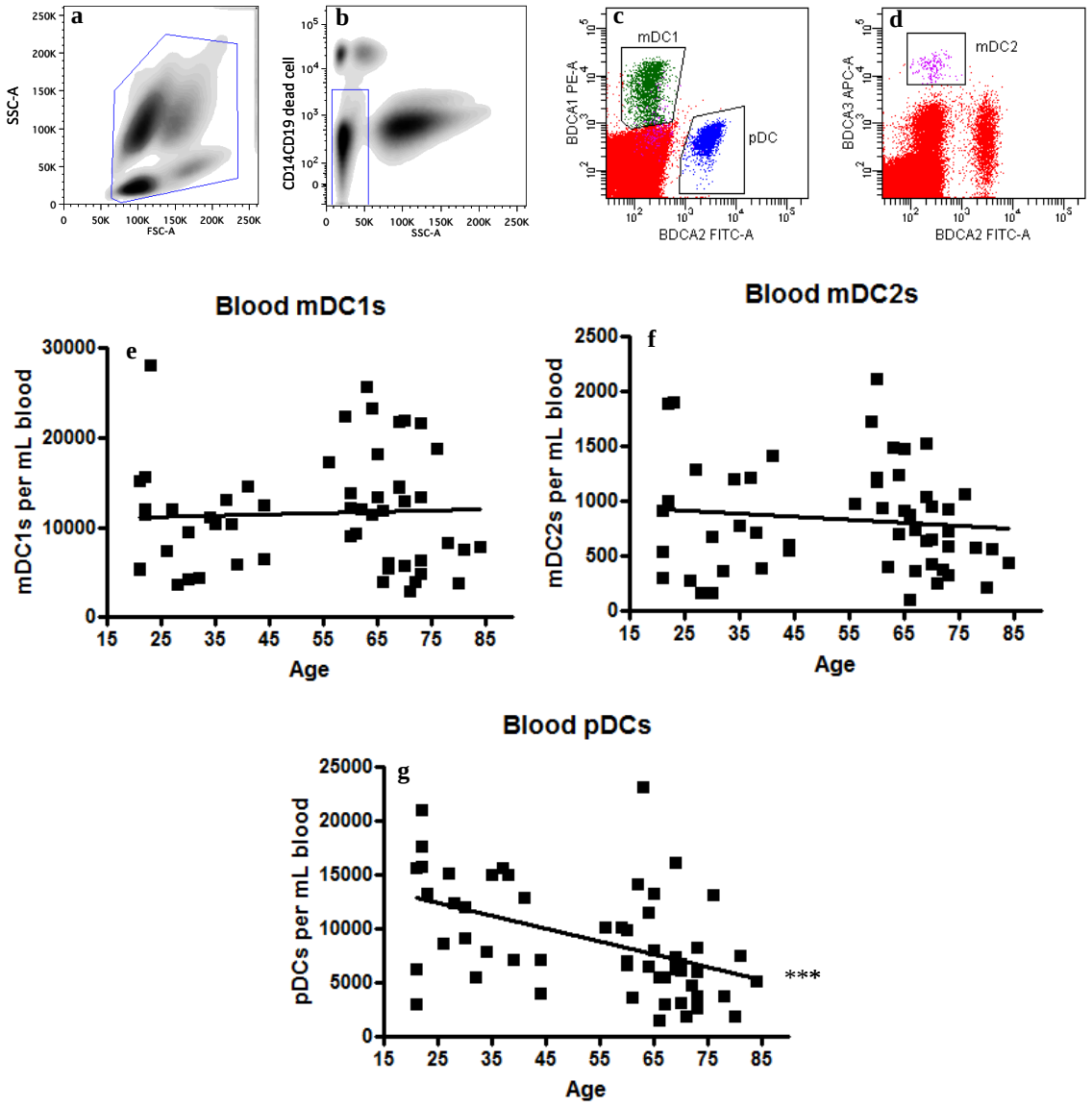

Figure 3.1: Plasmacytoid dendritic cell numbers decrease with age

Whole blood was stained for blood DC subpopulations and analysed by flow cytometry. Representative dot plot (a) showing gating of leukocytes by size and granularity. Monocytes, B-cells and granulocytes were further excluded by gating (b). Blood DC subpopulations were identified by high expression of BDCA-1 (c: mDC1), BDCA-3 (d: mDC2 and BDCA2 (c: pDC). The absolute number of circulating, myeloid dendritic cells (mDC1: e, and mDC2 : f) and plasmacytoid dendritic cells (pDC; g) measured as the number of DCs per ml of blood plotted against age. Absolute counts were determined by multiplying the number of DCs in the leukocyte gate by the number of PBMCs determined by counting on a haemocytometer. Each dot represent an individual volunteer (n = 54). P-values were determined using the two-tailed Mann-Whitney test. The solid line is the linear regression of continuous samples ***$p < 0.001$

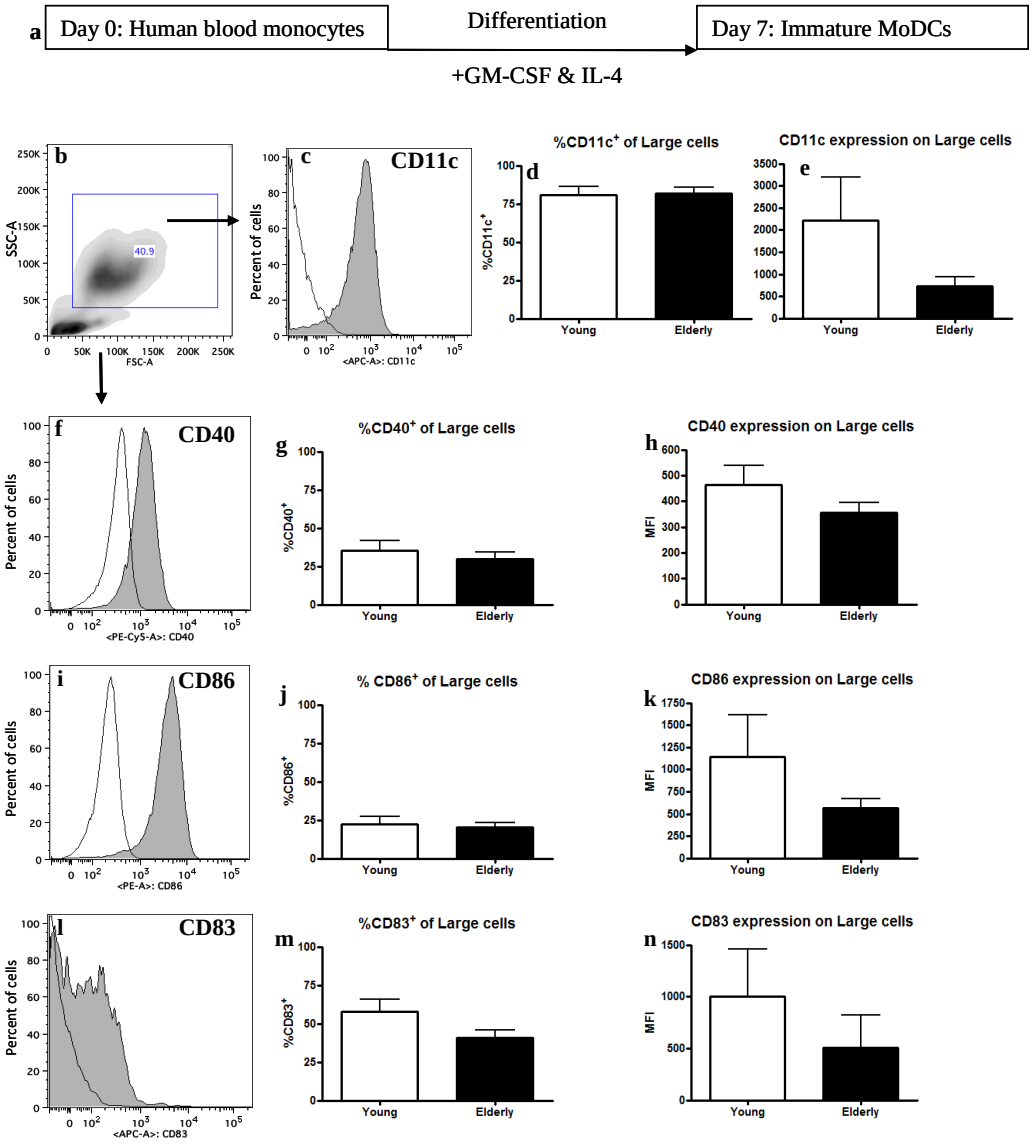

Figure 3.2: The capacity for monocytes to differentiate into iMoDCs is not significantly impaired with age

Human monocytes were differentiated into iMoDCs using GM-CSF and IL-4 (a). Immature MoDCs from healthy young and elderly volunteers were collected and cell surface molecules analysed by flow cytometry. Representative plot (b) showing gating of large cells which were analysed for expression of CD11c (c), CD40 (f), CD86 (i) and CD83 (l) with positive stained cells (grey filled) and unstained cells (unfilled). Pooled data of the percentages of cells positive for CD11c (d), CD40 (g), CD86 (j) and CD83 (m). Surface expression levels were measured and shown as MFIs of CD11c (e), CD40 (h), CD86 (k) and CD83 (n) in young (n = 19) versus elderly (n = 26) iMoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

variation meant that the differences did not reach statistical significance ($p = 0.28$). The statistical tests used were the two-tailed Mann-Whitney test of means of two groups and linear regression of continuous data.

CD40 (Figures 3.2f, 3.2g and 3.2h) and CD86 (Figures 3.2i, 3.2j and 3.2k) are important co-stimulatory molecules involved in T cell to DC interactions. CD83 (Figures 3.2l, 3.2m and 3.2n) is a key dendritic cell maturation marker (Figures 3.2c, 3.2f, 3.2i and 3.2l). Whilst the percentage of cells positive for CD40 (Figure 3.2g; $p = 0.58$) and CD86 (Figure 3.2j; $p = 0.99$) did not change between young and elderly volunteers, a decreasing trend with age was seen with the percent of cells positive for CD83 ($p = 0.068$; Figure 3.2m). Similarly, a decreasing trend with age was seen for surface expression levels of CD40 (Figure 3.2h: $p = 0.28$), CD86 (Figure 3.2k: $p = 0.26$) and CD83 (Figure 3.2n: $p = 0.052$). However again, individual variation meant that the differences did not reach statistical significance. Nonetheless, the data implies a reduced capacity to induce T cell activation by elderly dendritic cells. Again, the statistical tests used were the two-tailed Mann-Whitney test of means of two groups and linear regression of continuous data.

3.2.4 Age does not affect differentiation into immature pro-inflammatory $CD11c^+CD1a^+$ DCs

Myeloid DCs can differentiate along two independent pathways that give rise to $CD11c^+$ blood precursors that may or may not express membrane CD1a (Caux et al., 1996, Shortman and Liu, 2002). There is evidence that $CD11c^+CD1a^+$ DCs are functionally different to $CD11c^+CD1a^-$ DCs (Banchereau et al., 2000, Caux et al., 1997). In particular, immature $CD1a^+$ MoDCs secrete high amounts of IL-12p70 and less IL-10 than $CD1a^-$ DCs (Gogolak et al., 2007) implying an important pro-inflammatory role. Thus, the ability of $CD11c^+$ MoDCs to differentiate into immature $CD11c^+CD1a^+$ DCs in young versus elderly healthy volunteers was also examined. To do this $CD14^-$ cells were identified and $CD11c^+$ DCs gated (Figures 3.3a and 3.3b), and the percentage of cells positive (Figures 3.3c and 3.3d) and surface expression levels (MFIs) of $CD1a^+$ of $CD11c^+$ DCs (Figures 3.3c and 3.3e) measured. Whilst no significant difference was

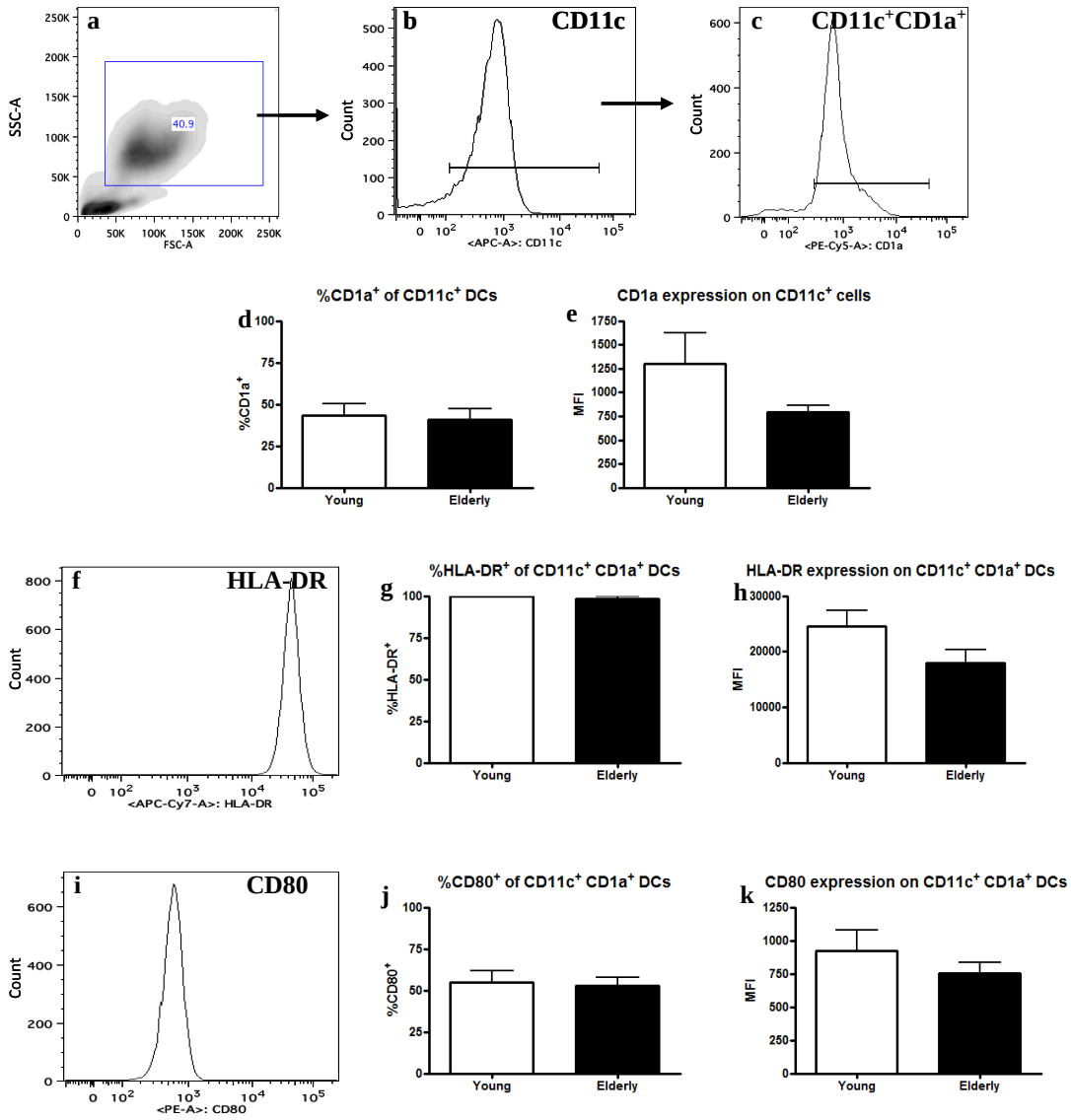

Figure 3.3: Monocytes readily differentiate into CD11c$^+$CD1a$^+$ iMoDCs regardless of age

Immature MoDCs from healthy young and elderly volunteers were collected and cell surface molecules analysed by flow cytometry. Large cells (a) were identified and gated on CD11c$^+$ cells (b). CD11c$^+$ DCs were further analysed and gated on CD1a$^+$ cells (c). CD11c$^+$CD1a$^+$ DCs were analysed for expression of HLA-DR (f) and CD80 (i). Pooled data for the percentage of cells positive for CD1a (d), HLA-DR (g) and CD80 (j) and for cell surface expression levels (MFIs) of CD1a (e), HLA-DR (h) and CD80 (k) is from young (n = 19) versus elderly (n = 26) iMoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

observed between the percentage of cells positive in MODCs from young and elderly volunteers (Figure 3.3d; p = 0.70), a trend towards decreased surface level expression was seen with age for CD1a however, the differences did not reach statistical significance (Figure 3.3e; p = 0.17).

$CD1a^{+}CD11c^{+}$ DCs were then examined for expression of HLA-DR (a MHC class II molecule involved in antigen presentation to $CD4^{+}$ T cells) and CD80 (another co-stimulatory molecule involved in T-cell to DC interaction) (Figures 3.3f and 3.3i). Whilst no significant difference was seen between young and elderly volunteers in the percentage of cells positive for HLA-DR (Figure 3.3g; p = 1.00), CD80 (Figure 3.3j; p = 0.67), or surface molecule expression levels of CD80 (Figure 3.3k; p = 0.56), a decreased trend with age was seen for HLA-DR surface expression levels (Figure 3.3h; p = 0.061).

3.2.5 Age does not impact on the antigen processing ability of iMODCs.

The primary role of immature DCs is to take up, internalize and process antigen, which triggers their maturation. Once mature, DCs lose their ability to process antigen. Thus, the next series of experiments compared the antigen processing ability of iMoDCs prepared from 16 healthy young versus 28 elderly volunteers using the DQ-Ovalbumin (DQ-OVA) assay. The DQ-OVA assay measures antigen processing by the fluorescence of a self-quenching ovalbumin conjugate following proteolytic degradation. Immature MoDCs were first gated for by size (Figure 3.4a) and $CD14^{-}$ cells examined for the degradation of $FITC^{+}$DQ-Ovalbumin indicating antigen processing (Figure 3.4b). Samples were analysed for the percentage of $CD14^{-}$ cells able to process antigen (Figure 3.4c) and the level of antigen processing (MFI; Figure 3.4d). No significant differences were seen in antigen processing ability between the two age groups (Figure 3.4c: p = 0.14; Figure 3.4d: p = 0.41).

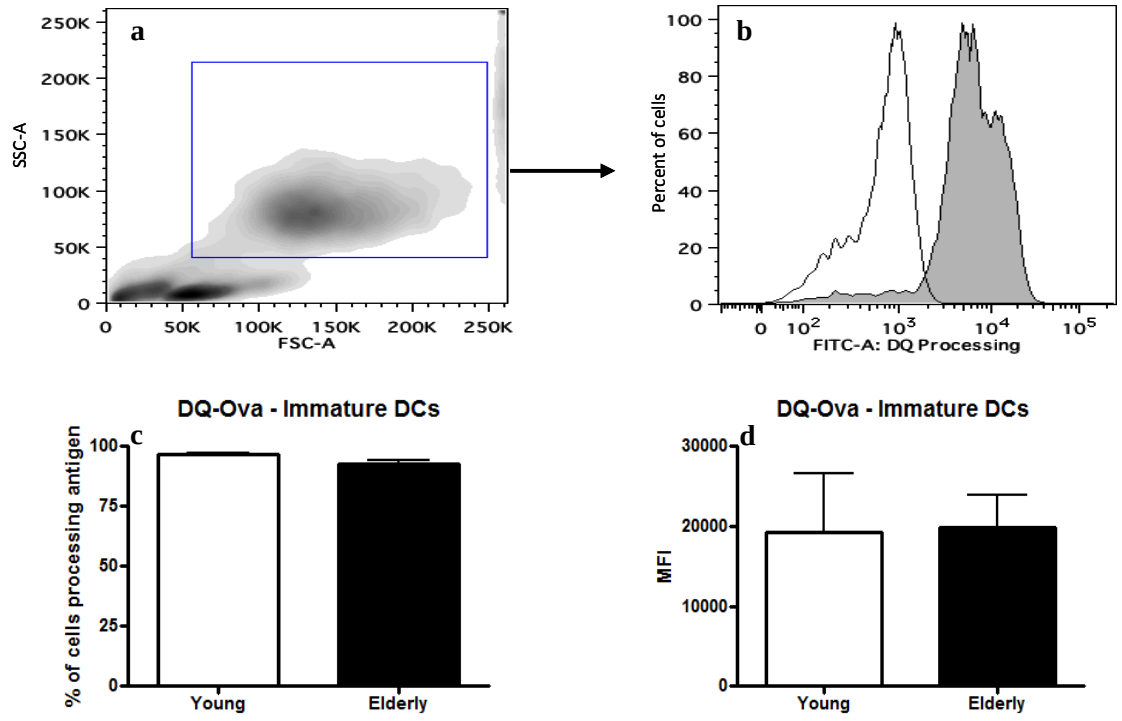

Figure 3.4: Age has no impact on the antigen processing ability of immature MoDCs

Immature MoDCs from healthy young and elderly individuals were incubated for 1 hour with DQ-Ovalbumin. Representative dot plot (a) showing gating of MoDCs based on size and granularity. The capacity to process antigen was determined by emission of a signal in the FITC channel (b) and measured by flow cytometry; red histogram represents cells incubated with DQ-OVA, grey histogram represents control cells that did not receive DQ-OVA. Pooled data (c) indicating % of immature DCs able to process antigen and (d) mean fluorescent intensity (MFI) indicating relative antigen processing capacity of elderly (n = 28) versus young (n = 16) iMoDCs. Pooled data is shown as mean ± SEM.

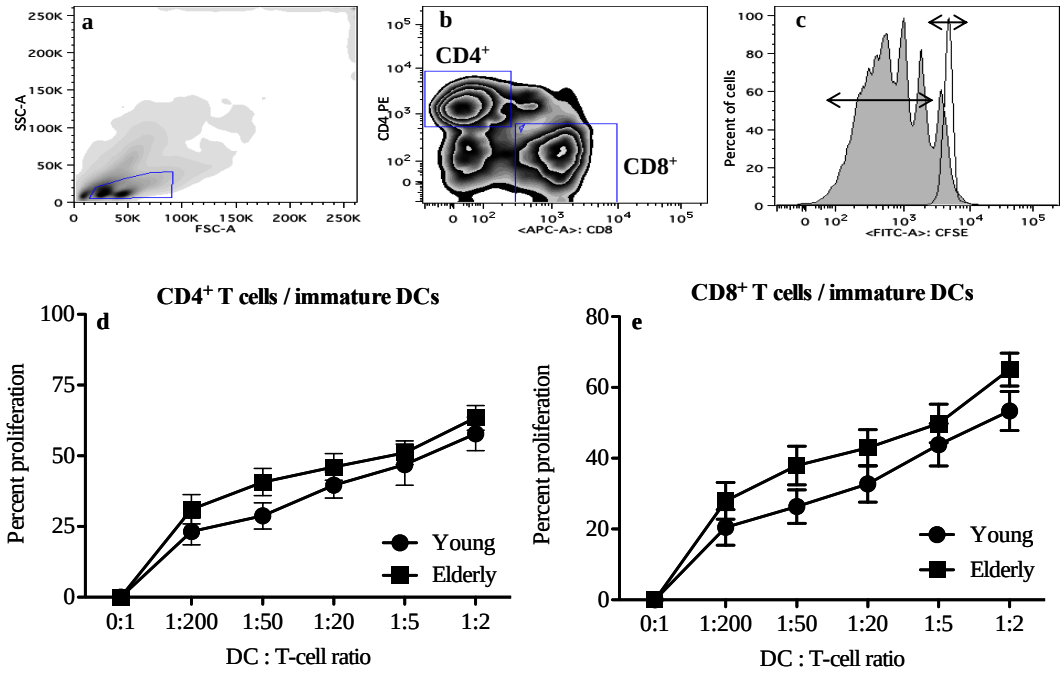

Figure 3.5: Age does not modulate the ability of iMoDCs to induce lymphocyte proliferation

Immature MoDCs were co-cultured with allogeneic CFSE-labelled lymphocytes for 7 days. Cells were collected and stained for CD4 and CD8 expression and analysed by flow cytometry. Representative plot (a) showing gating of lymphocytes by size and granularity. Lymphocytes were further gated as either $CD4^+$ or $CD8^+$ (b). The percentage of proliferating cells of the total gated population (longer arrow) was determined; the smaller arrow shows the non-proliferating parent peak. Pooled percentage proliferation was plotted against DC:T-cell ratio for $CD4^+$ (d) and $CD8^+$ (e) T cells in young (n = 16) versus elderly (n = 19) iMoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

3.2.1 Ages does not affect the ability of iMoDCs to induce T lymphocyte proliferation

The primary function of mature DCs is to present antigen to T cells, whereas immature DCs are poor antigen presenters. To investigate whether aging alters the capacity of immature DCs to present antigen we used the allogeneic mixed lymphocyte reaction (MLR) assay. Briefly MoDCs from 35 healthy individuals were co-cultured with allogeneic CFSE-labelled lymphocytes from a universal young healthy male donor aged 34. Lymphocytes were first gated by size (Figure 3.5a) and further gated into $CD4^+$ and $CD8^+$ lymphocytes (Figure 3.5b). Lymphocyte proliferation was identified by a decrease of CFSE due to dilution between daughter cells. Proliferation was determined by the difference in parent population compared to unstimulated lymphocytes (Figure 3.5c). Although iMoDCs from elderly volunteers demonstrated a slightly increased ability to induce proliferation of $CD4^+$ or $CD8^+$ lymphocytes, possibly indicating a slightly more mature state, the difference was not statistically significant (Figures 3.5d and 3.5e).

3.2.2 CD83 expression in elderly-derived LPS-activated MoDCs is reduced

DCs mature in response to various stimuli including signals from cytokines such as IFNγ, and as well as signals from microbial components such as lipopolysaccharide (LPS). DCs can rapidly respond to these signals by up-regulating expression of cell surface markers such as CD11c, CD40, CD80, CD86 CD83 and HLA-DR. To investigate whether this response is modulated during aging, iMoDCs from healthy volunteers were exposed to LPS or IFNγ, or both, for 48 hours.

Firstly, iMoDCs from 45 healthy volunteers were exposed to LPS for 48 hours (Figure 3.6a). Cells were gated by size (as shown in Figure 3.2) and $CD14^-$ cells investigated for surface expression levels and percentage cells positive of CD11c (Figures 3.6b and 3.6c,), CD40 (Figures 3.6d and 3.6e), CD86 (Figures 3.6f and 3.6g) and CD83 (Figures 3.6h and 3.6i). There were no differences observed in the percentage of cells positive for CD11c (Figure 3.6b; $p = 0.97$), CD40 (Figure 3.6d; $p = 0.97$), CD86 (Figure 3.6f; $p = 0.23$) and CD83 (Figure 3.6h; $p = 0.12$). Whilst there was no significant difference in surface expression levels (MFI) for CD40 (Figure 3.6e; $p = 0.49$) and CD86 (Figure

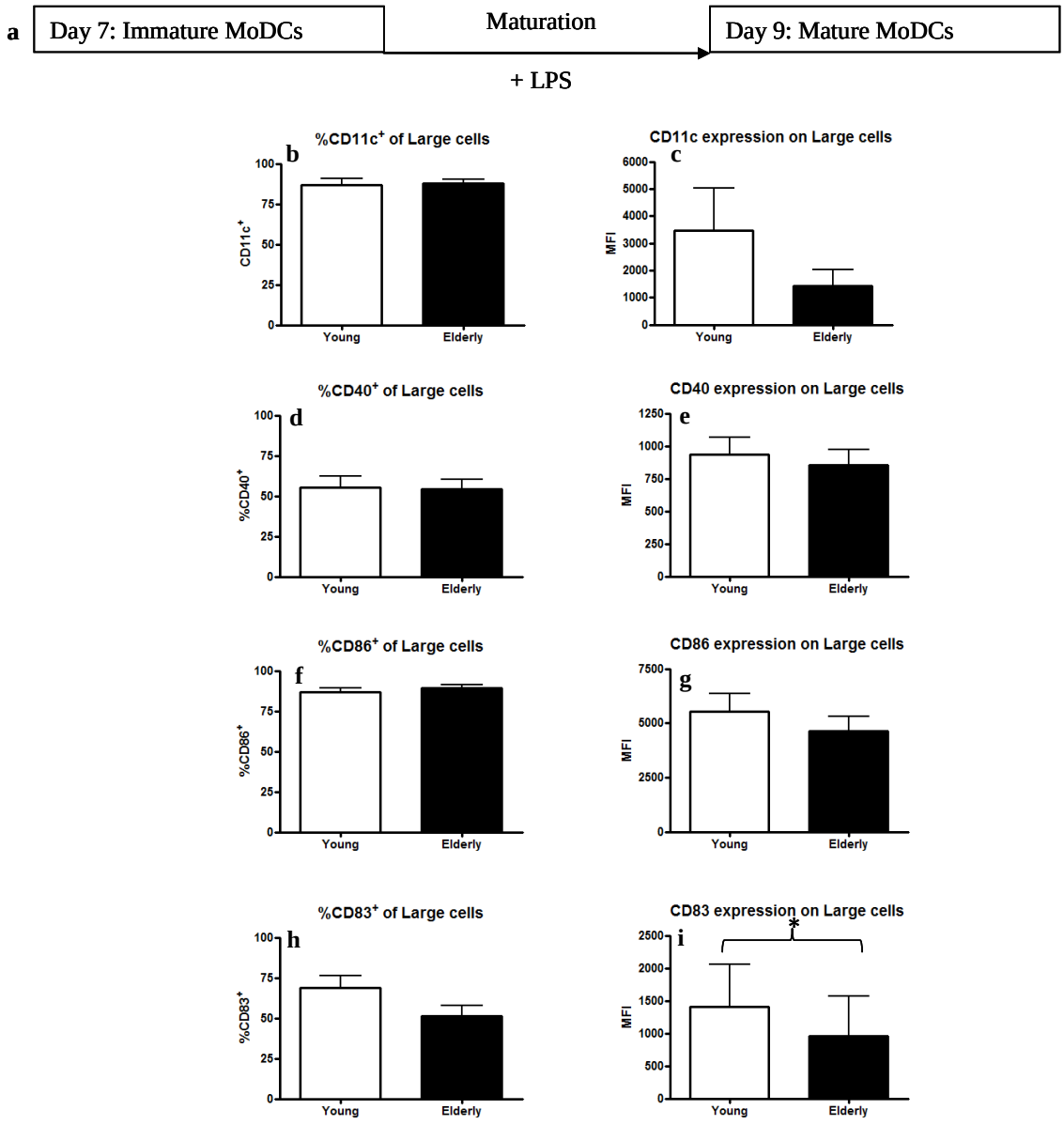

Figure 3.6: CD83 expression in elderly LPS activated MoDCs is reduced compared to young MoDCs

Immature MoDCs generated from young and elderly volunteers were stimulated with LPS (a) and cell surface molecules analysed by flow cytometry for expression of CD11c (b,c), CD40 (d,e), CD86 (f,g) and CD83 (h,i). Pooled data of the percentages of cells positive for CD11c (b), CD40 (d), CD86 (f) and CD83 (h). Surface expression levels were measured and shown as MFIs of CD11c (c), CD40 (e), CD86 (g) and CD83 (i) in young (n = 19) versus elderly (n = 26) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test. *$p < 0.05$

3.6g; p = 0.33), a decreasing trend with age was observed for CD11c (Figure 3.6c; p = 0.18) and a significant decrease with age was observed for CD83 (Figure 3.6i; p = 0.025).

The LPS matured $CD14^-$ cells were further gated as $CD11c^+$ then as $CD1a^+CD11c^+$ DCs (as shown in Figure 3.3). Whilst there was no significant difference for the percent $CD11c^+$ cells that differentiated into $CD1a^+$ DCs (Figure 3.7a; p = 0.35) there was an age-related trend towards decreased surface expression levels of CD1a with increased age (Figure 3.7b; p = 0.19). As previously described, $CD1a^+CD11c^+$ DCs were also examined for the percentage of cells positive and surface expression levels of HLA-DR (Figures 3.7c and 3.7d) and CD80 (Figures 3.7e and 3.7f). No statistically significant differences according to age were seen for the percentage of cells positive for HLA-DR (Figure 3.7c; p = 0.64), CD80 (Figure 3.7e; p = 0.60) or CD80 expression levels (Figure 3.7f; p = 0.26). However, a trend towards decreased surface HLA-DR expression levels was observed with increasing age (Figure 3.7d; p = 0.061).

3.2.3 MoDCs from elderly volunteers do not fully mature by losing their antigen processing capacity

The next experiments further examined whether MoDCs from elderly volunteers matured in response to LPS stimuli by losing their capacity to process antigen; measured using the DQ-OVA assay (described in Figure 3.4). One hundred percent of iMoDCs from young volunteers readily processed antigen (refer to Figure 3.4c) however, 80% of these cells lost this function after maturation with LPS (Figures 3.8a and 3.8c). A two-fold reduction of expression levels (MFI) in the cells that were positive for $FITC^+$DQ-OVA also confirm loss of antigen processing capacity (refer to Figure 3.4d and Figures 3.8a and 3.8d) in association with maturation. In contrast, >45% of LPS-matured MoDCs from the elderly retained their antigen processing ability, however the amounts (MFIs) of DQ-OVA internalized were reduced (Figures 3.8b, 3.8c; p = 0.13 and 3.8d; p = 0.71). These data imply that whilst MoDCs from the elderly respond to LPS they do not mature at equivalent levels to those from younger subjects.

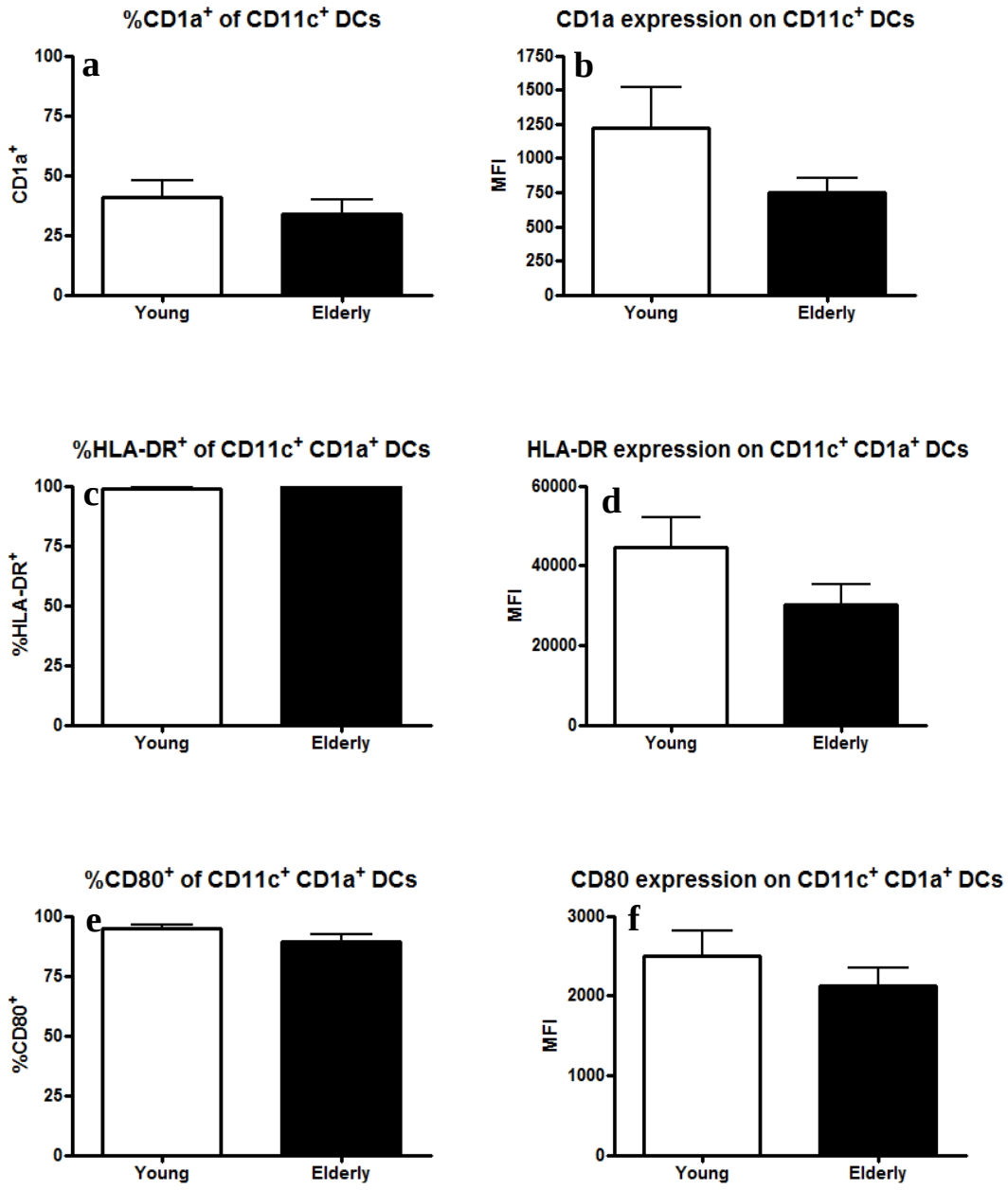

Figure 3.7: CD1a, HLA-DR and CD80 on LPS-stimulated CD11c$^+$ MoDCs is not affected by age

Immature MoDCs generated from young and elderly volunteers were stimulated with LPS and cell surface molecules analysed by flow cytometry. Large cells were identified and gated on CD11c$^+$ cells. Pooled data for the percentage of CD11c$^+$ cells positive for CD1a (a), HLA-DR (c) and CD80 (e) and for cell surface expression levels (MFIs) of CD1a (b), HLA-DR (d) and CD80 (f) is from young (n = 19) versus elderly (n = 26) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

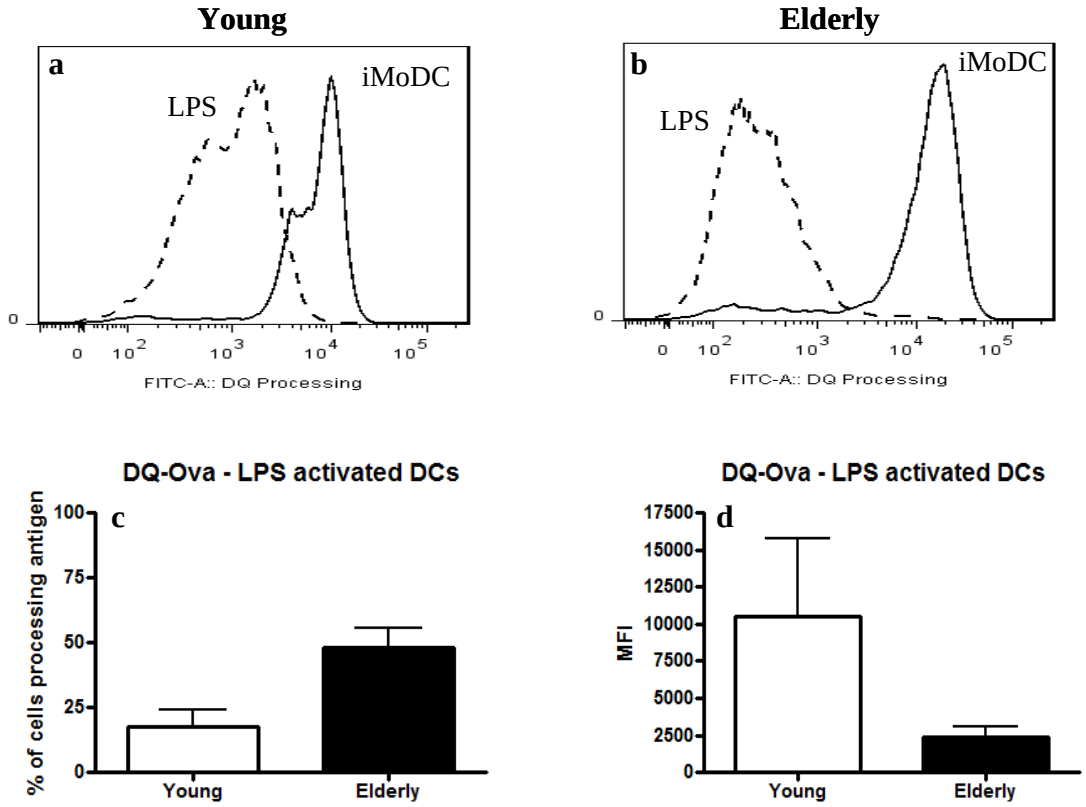

Figure 3.8: LPS-matured MoDCs from elderly volunteers maintain their capacity to process antigen

MoDCs from healthy young and elderly individuals previously stimulated with LPS or IFNγ were incubated for 1 hour with FITC-DQ-Ovalbumin (DQ-OVA) as per Figure 4. Representative histograms from young (a) versus elderly (b) derived iMoDCs and LPS-matured MoDCs. Pooled data of the percent of DCs still processing antigen (c), MFIs indicating relative antigen processing capacity (d) from LPS-treated elderly (n = 25) versus young (n = 12) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

3.2.1 LPS-matured-MoDCs from elderly volunteers readily induce CD8 proliferation

Maturation of DCs leads to an increased ability to present antigen to T cells. Thus, LPS matured MoDCs from 32 healthy individuals were examined for their ability to induce lymphocytes from the universal allogeneic donor to proliferate. Lymphocytes were identified by size (Figure 3.9a), and T cell subsets identified by CD4 or CD8 expression (as shown in Figures 3.9a, 3.9b and 3.9c). Whilst no differences were observed for $CD4^+$ T cell proliferation (Figure 3.9d), a significant increase was seen for $CD8^+$ T cell proliferation induced by MoDCs from elderly subjects when the DC:Lymphocyte ratio was less than or equal to 1:50 (Figure 3.9e; 1:200 $p = 0.042$, 1:50 $p = 0.024$).

3.2.2 Age does not modulate cytokine secretion in LPS-activated MoDCs

An important function of mature DCs is their ability to secrete various cytokines that can regulate adaptive immunity. Thus, TNF, IL-10, VEGF, IL-12p70 and IFNγ in media from LPS-activated MoDCs were measured using a cytometric bead array. Beads were identified by size (Figure 3.10a), then individual cytokine-specific beads gated for based on expression of APC and APC-Cy7 (Figure 3.10b) and cytokine concentrations determined by PE MFIs (Figure 3.10c). None of the cytokines were secreted by iMoDCs, in contrast detectable cytokines were detected in all LPS-activated MoDCs and no differences were seen between LPS-activated MoDCs from young and elderly individuals (Figure 3.10e; TNFα: $p = 0.98$, Figure 3.10f; IL-10: $p = 0.68$, Figure 3.10g; VEGF: $p = 0.89$, Figure 3.10h; IL-12p70: $p = 0.27$ and Figure 3.10i; IFNγ: $p = 0.089$).

3.2.3 IFNγ activation increases CD86 and CD80 expression in elderly-derived MoDCs

Immature iMoDCs were also exposed to IFNγ for 48 hours prior to analysis (Figure 3.11a). No significant differences between age groups were seen in $CD14^-$ gated cells examined for the percentage of $CD11c^+$ cells (Figure 3.11b; $p = 0.83$) or for CD11c surface expression levels (Figure 3.11c; $p = 0.31$).

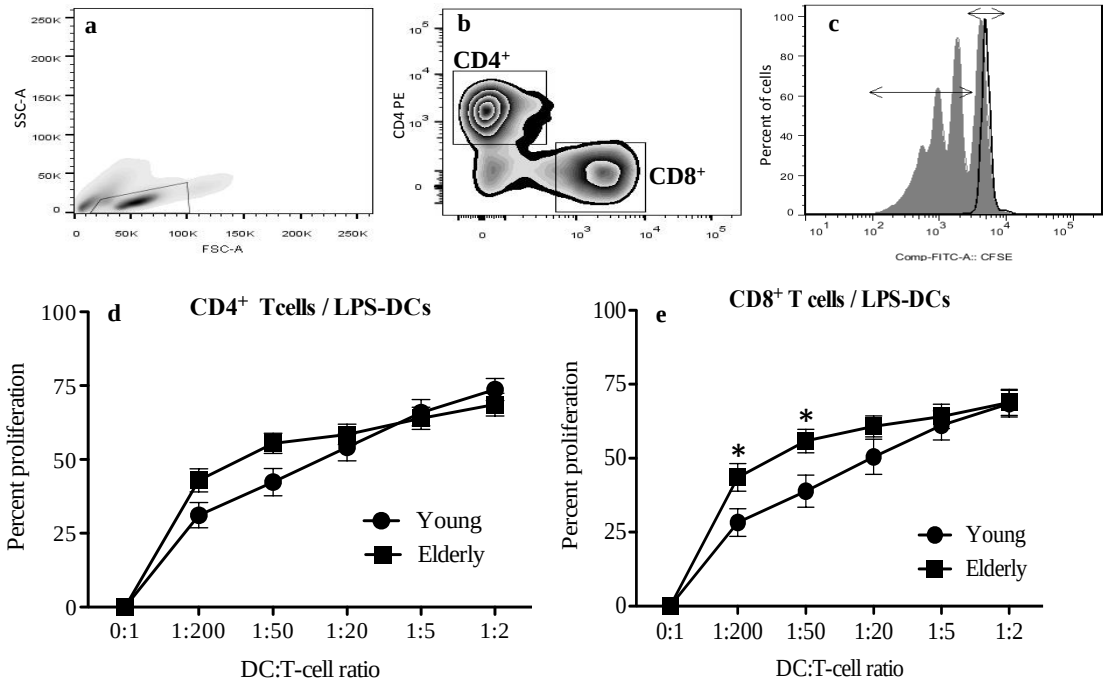

Figure 3.9: Aging improves the ability of LPS-matured MoDCs to induce CD8 proliferation

LPS-activated MoDCs were co-cultured with allogeneic CFSE labelled lymphocytes for 7 days. Cells were collected, stained for CD4 and CD8 expression and analysed by flow cytometry. Representative plot (a) showing gating of lymphocytes by size and granularity. Lymphocytes were further gated as either $CD4^+$ or $CD8^+$ (b). The percentage of proliferating cells of the total gated population (longer arrow) was determined; the smaller arrow shows the non-proliferating parent peak. Pooled percentage proliferation was plotted against DC: T-cell ratio for young (n = 13) versus elderly (n = 19) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

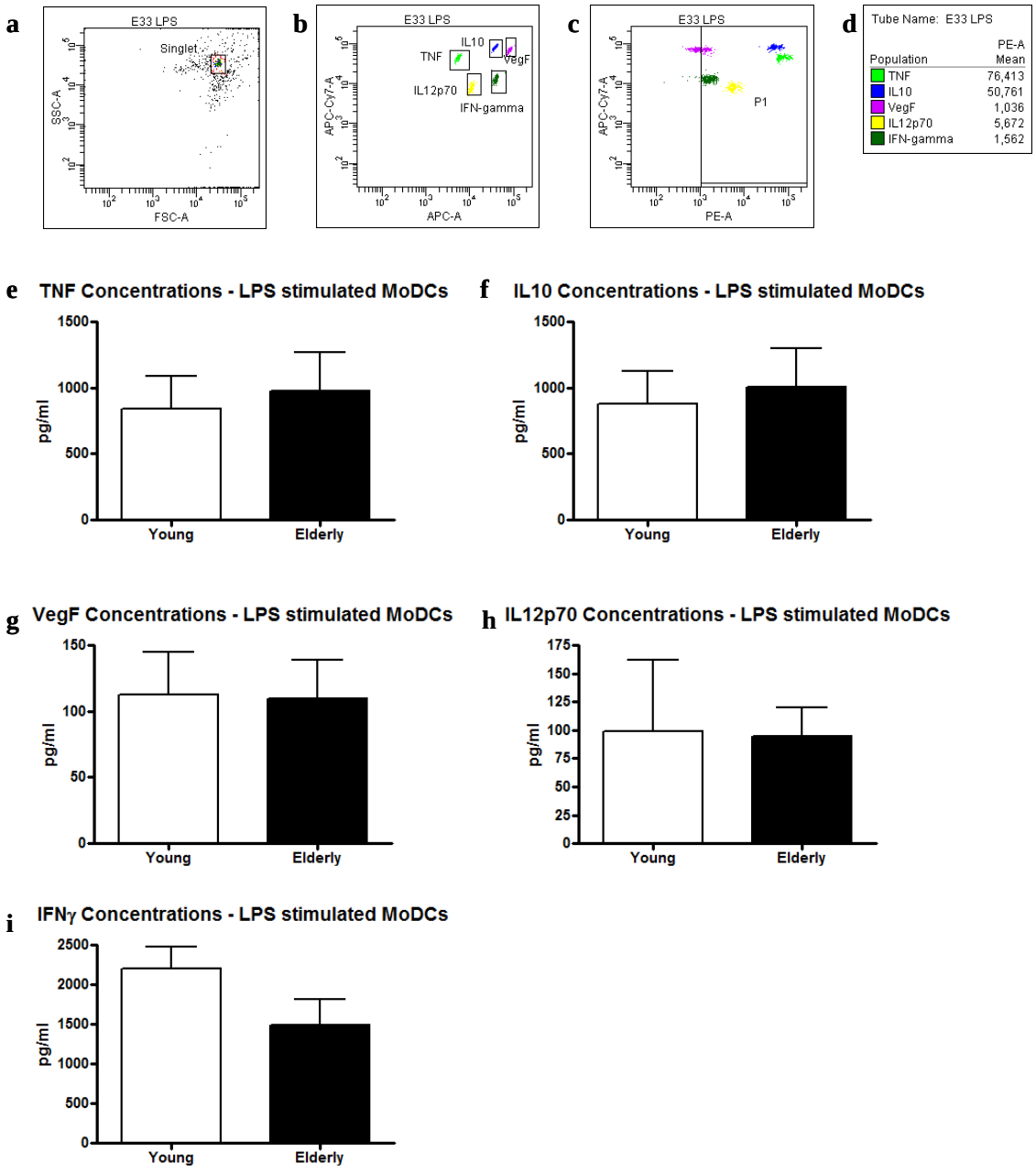

Figure 3.10: Age does not affect cytokine secretion in MoDCs activated by LPS

Culture media from LPS-stimulated MoDCs from healthy young and elderly volunteers were analysed by cytometric bead array for the production of cytokines. Representative plot (a) showing gating of pooled beads by size and granularity. Beads were further gated (b) to identify each cytokine tested. Concentration of cytokine present was determine by measuring MFI (c,d) and correlating it to a standard curve. Pooled data for the concentration of TNF (e), IL-10 (f), VEGF (g), IL-12p70 (h) and IFNγ (i) present in culture media containing young (n = 11) and elderly (n = 14) MoDCs. Pooled data is shown as mean ±SEM.

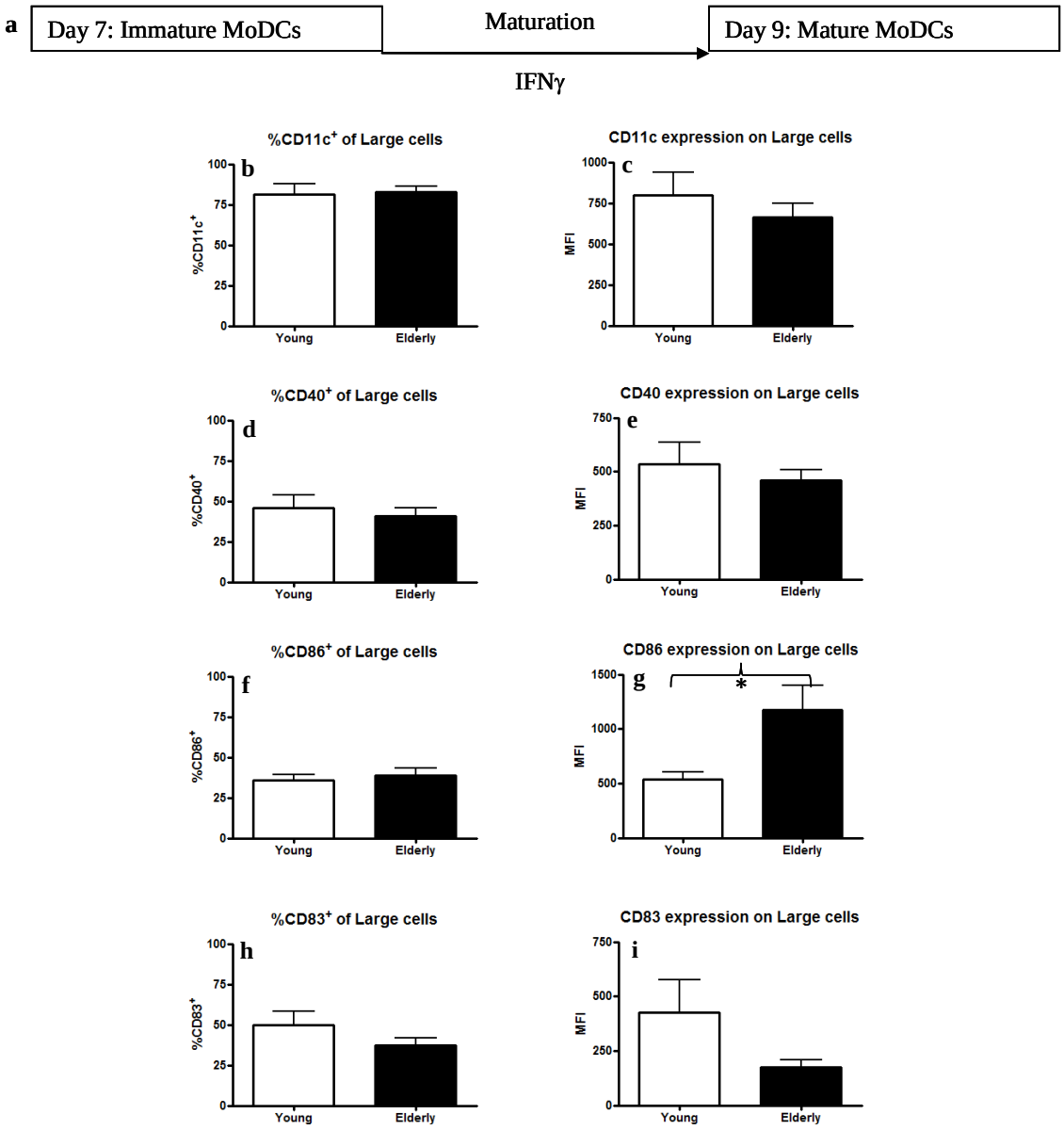

Figure 3.11: CD86 expression in elderly IFNγ-activated MoDCs is increased compared to young MoDCs

Immature MoDCs generated from young and elderly volunteers were stimulated with IFNγ (a) and cell surface molecules analysed by flow cytometry. Large cells were analysed for expression of CD11c (b,c), CD40 (d,e), CD86 (f,g) and CD83 (h,i). Pooled data of the percentages of cells positive for CD11c (b), CD40 (d), CD86 (f) and CD83 (h). Surface expression levels were measured and shown as MFIs of CD11c (c), CD40 (e), CD86 (g) and CD83 (i) in young (n = 13) versus elderly (n = 25) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test. *$p < 0.05$

Similarly, no significant differences were seen for the percentage of $CD14^-$ cells positive for CD40 (Figure 3.11d; p = 0.44), CD86 (Figure 3.11f; p = 0.81) and CD83 (Figure 3.11h; p = 0.20). Furthermore, no differences in surface expression levels were seen for CD40 (Figure 3.11e; p = 0.76). A trend for a decrease with age was observed for CD83 surface expression levels (Figure 3.11i; p = 0.10). In contrast, CD86 surface expression levels were significantly increased in elderly participants (Figure 3.11g; p = 0.015).

IFNγ-activated $CD14^-CD1a^+CD11c^+$ MoDCs cells were also examined. No significant differences between the two age groups were observed for the percentage of $CD11c^+$ cells that differentiated into $CD1a^+$ DCs (Figure 3.12a; p = 0.85). $CD1a^+$ expression levels were also similar (Figure 3.12b; p = 0.58). No differences were observed for the percent of cells positive for $HLA\text{-}DR^+$ on $CD1a^+CD11c^+$ DCs (Figure 3.12c; p = 0.31) however, a decreased trend was observed for surface HLA-DR expression (Figure 3.12d; p = 0.36). In contrast, a trend towards an increase with age was seen for both the percentage of $CD80^+$ cells (Figure 3.12e; p = 0.074) and for CD80 surface expression levels (Figure 3.12f; p = 0.18).

3.2.4 IFNγ stimulation reveals maturation paralysis in MoDCs from elderly volunteers

Greater than 70% of IFNγ-stimulated MoDCs from young and elderly participants were still able to process $FITC^+$DQ-OVA (Figures 3.13a, 3.13b and 3.13c). However, whilst young-derived MoDCs drastically down regulated their antigen processing capacity elderly-derived MoDCs contained significantly higher levels of DQ-OVA (Figure 3.13d; p = 0.012). Thus, elderly-derived MoDCs maintained their antigen processing capacity and did not mature appropriately after IFNγ stimulation implying a significant defect in their responses to IFNγ.

IFNγ-activated MoDCs show an increased trend to induce T cell proliferation with age

IFN-γ-matured MoDCs from 18 healthy volunteers were examined for their ability to induce T-cells from the universal allogeneic young donor to proliferate. T lymphocytes were gated firstly by size, then by expression of CD4 and CD8 (as shown in Figure 3.9).

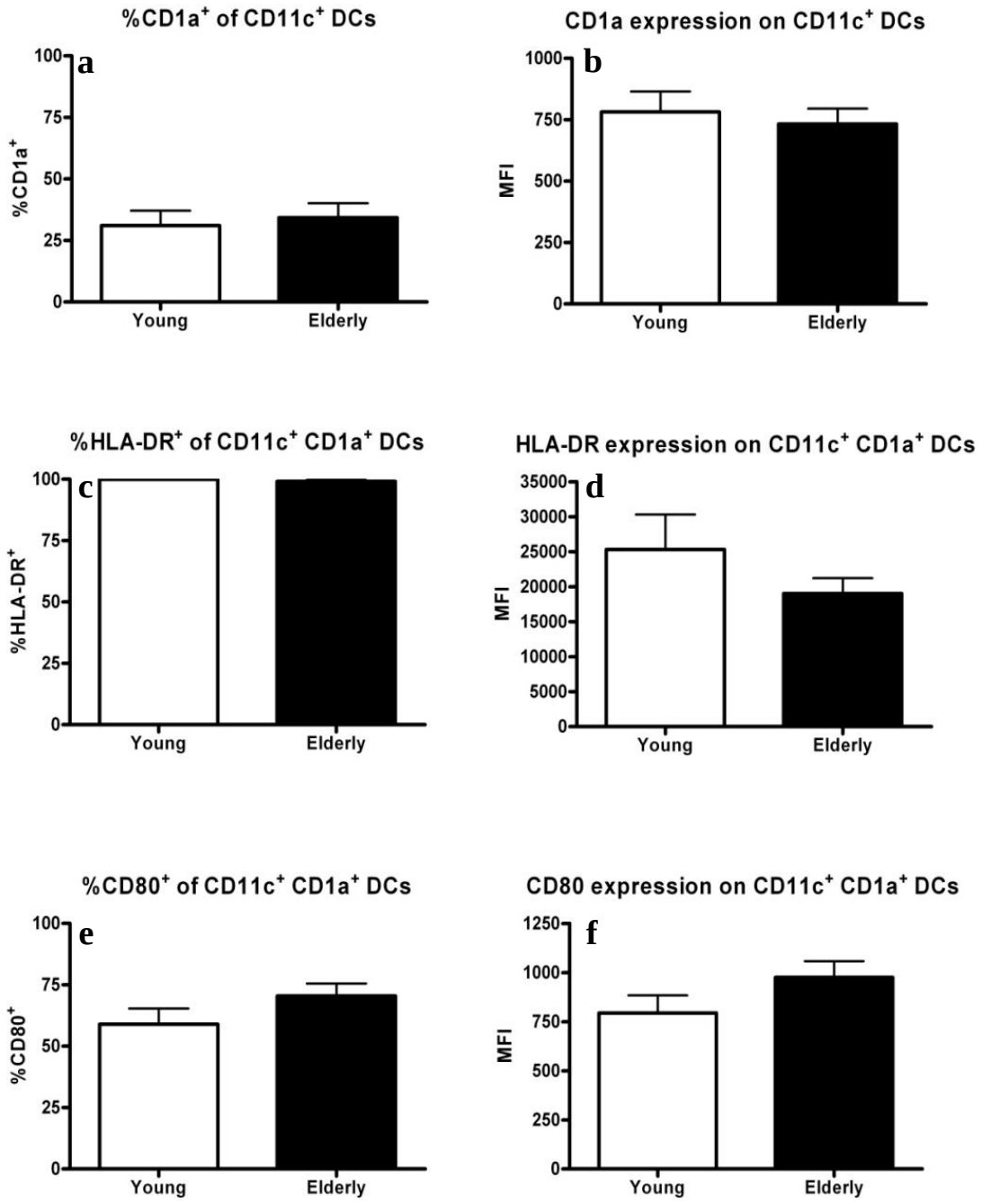

Figure 3.12: CD1a, HLA-DR and CD80 on IFNγ-stimulated CD11c$^+$ MoDCs is not affected by age

Immature MoDCs generated from young and elderly volunteers were stimulated with IFNγ and cell surface molecules analysed by flow cytometry after gating on CD11c$^+$ cells. CD11c$^+$ DCs were further gated on CD1a$^+$ cells (a and b). CD11c$^+$CD1a$^+$ DCs were analysed for expression of HLA-DR (c and d) and CD80 (e and f). Pooled data is from young (n = 13) versus elderly (n = 25) MoDCs and shown as mean ± SEM.

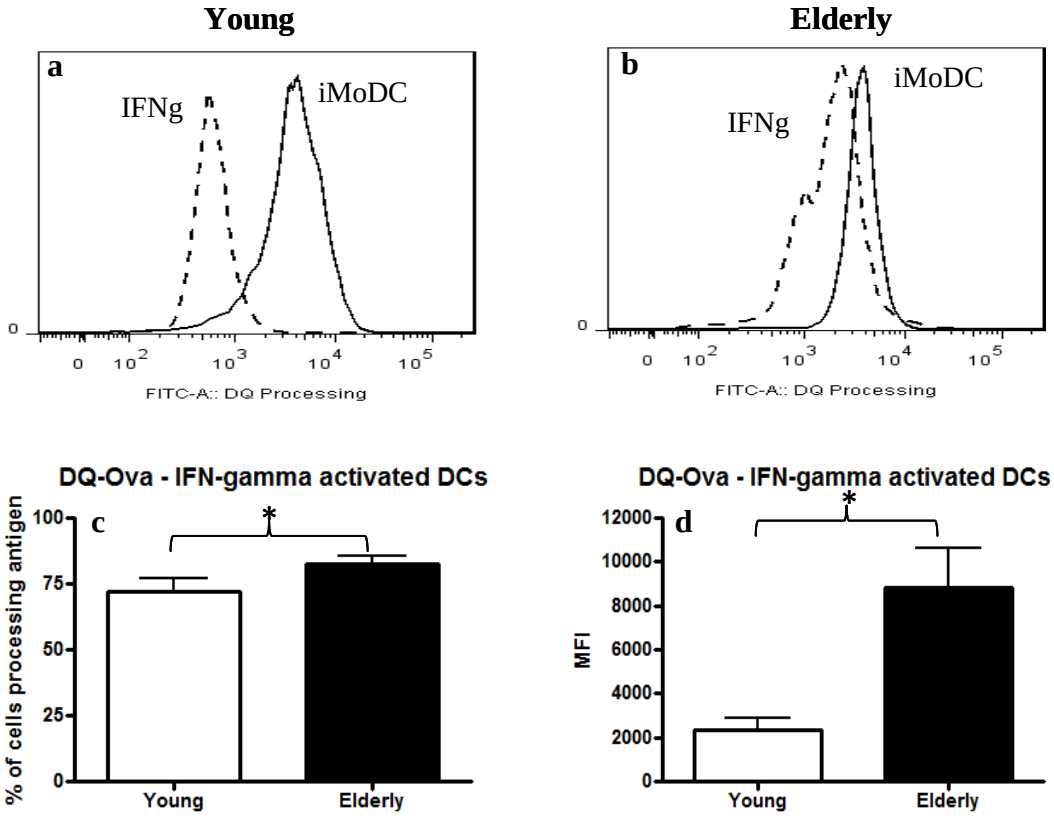

Figure 3.13: IFNγ-matured MoDCs from elderly volunteers maintain their capacity to process antigen

MoDCs from healthy young and elderly individuals previously stimulated with LPS or IFNγ were incubated for one hour with FITC-DQ-Ovalbumin (DQ-OVA) as per Figure 4. Representative histograms from young (a) versus elderly (b) derived iMoDCs and IFNγ-matured MoDCs. Pooled data showing the percent of DCs still processing antigen (c) or MFIs indicating relative antigen processing capacity (d) from IFNγ-treated elderly (n = 25) versus young (n = 12) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test. *$p < 0.05$

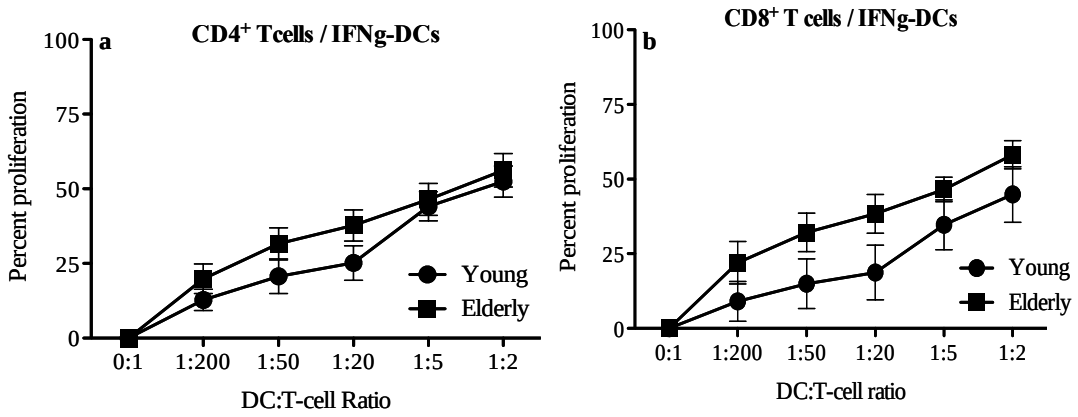

Figure 3.14: Age does not alter the capacity of IFNγ-matured DCs to induce $CD4^+$ T cell proliferation

IFNγ-activated MoDCs were co-cultured with allogeniec CFSE labelled lymphocytes for 7 days. Cells were collected, stained for CD4 and CD8 expression and analysed by flow cytometry.Lymphocytes were identified by size and granularity and further gated as either $CD4^+$ or $CD8^+$. The percentage of proliferating cells of the total gated population was determined. Pooled percentage proliferation was plotted against DC: T-cell ratio for for $CD4^+$ (a) and $CD8^+$ (b) T cells co-cultured with young (n = 6) versus elderly-derived (n = 12) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

MoDCs from elderly volunteers were at least as efficient if not better than the MoDCs from younger volunteers at driving CD4 and CD8 T-cell proliferation (Figures 3.14a and 3.14b).

3.2.5 Elderly-derived MoDCs produce more TNF than young-derived MoDCs following IFNγ activation

Following IFNγ-activation of MoDCs, culture media was collected and analysed for secreted cytokine content by cytometric bead array. Measured concentrations of IL-10, IL-12p70 and VEGF were below the detection limit of the assay for both young-derived and elderly-derived IFNγ-activated MoDCs (data not shown). No difference was observed in the secretion of IFNγ between elderly and young derived MoDCs (Figure 3.15b; $p = 0.78$). In contrast, a significant increase in TNF secretion was observed for elderly-derived MoDCs compared to young-derived MoDCs (Figure 3.15a; $p = 0.048$).

3.2.6 MoDCs generate equivalent phenotypic responses to LPS/IFNγ stimulation regardless of age

The data above revealed differential DC responses to LPS or IFNγ in MoDCs from elderly versus young subjects, although partial activation in elderly-derived MoDCs appeared to be induced by either stimulus. Therefore, MoDCs from young and elderly volunteers were activated with a combination of IFNγ and LPS for 48 hours (Figure 3.16a) and gated by size. No differences were observed between the two age groups for the percentage of $CD14^-$ cells that were $CD11c^+$ (Figure 3.16b; $p = 0.69$), $CD40^+$ (Figure 3.16d; $p = 0.49$), $CD86^+$ (Figure 3.16f; $p = 0.82$) and $CD83^+$ (Figure 3.16h; $p = 0.32$). Similarly, surface expression levels were not changed for CD11c (Figure 3.16c; $p = 0.95$), CD40 (Figure 3.16e; $p = 0.69$) and CD86 (Figure 3.16g; $p = 0.88$). In contrast, a decreased trend for CD83 (Figure 3.16i; $p = 0.12$) was seen with age.

IFN-γ/LPS activated MoDCs were further gated as $CD11c^+$ then as $CD11c^+CD1a^+$ DCs. There were no differences for the percent of $CD11c^+$ MoDCs that were $CD1a^+$ (Figure 3.17a; $p = 0.64$) or for $CD1a^+$ surface expression levels (Figure 3.17b; $p = 0.37$) between young and elderly volunteers. As previously, $CD11c^+CD1a^+$ DCs were further examined

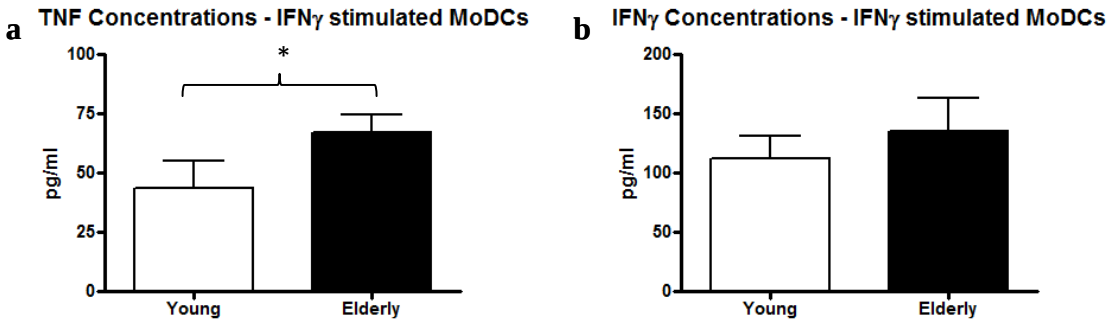

Figure 3.15: MoDCs from elderly individuals produce higher levels of TNF than MoDCs from young individuals following activation with IFNγ

Culture media from IFNγ-stimulated MoDCs from healthy young and elderly volunteers was analysed by cytometric bead array for the production of cytokines. Cytokine concentration was determined by measuring the MFI of the corresponding antibody-conjugated beads as per Figure 10. Pooled data for the concentration of TNF (a) and IFNγ (b) present in culture media containing young (n = 12) and elderly (n = 14) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test. *$p < 0.05$

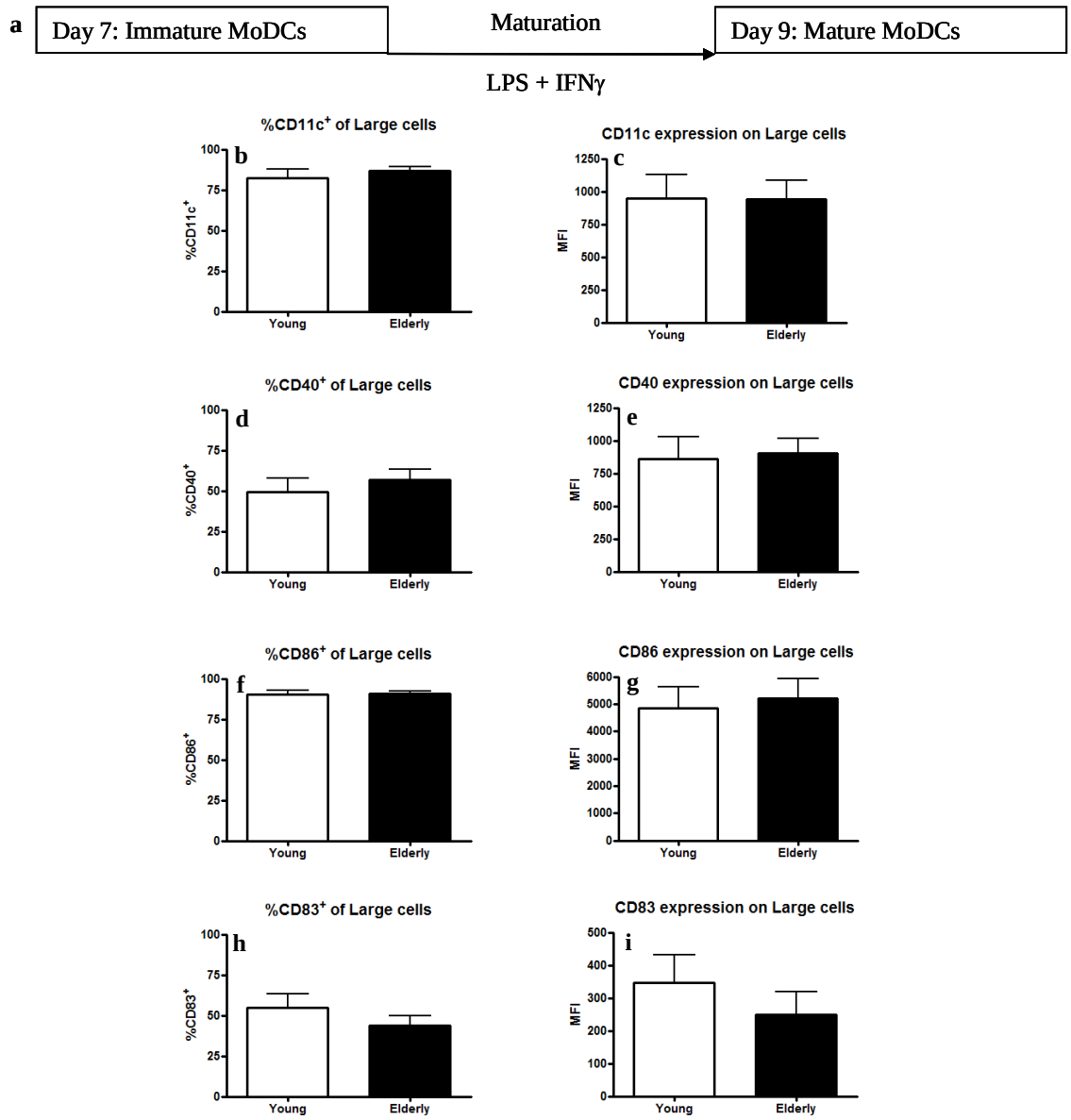

Figure 3.16: Age does not impair the capacity of LPS + IFNγ activated iMoDCs to mature

Immature MoDCs generated from young and elderly volunteers were stimulated with LPS and IFNγ (a) and cell surface molecules analysed by flow cytometry. Representative plot (b) showing gating of large cells which were analysed for expression of CD11c (c), CD40 (f), CD86 (i) and CD83 (l) with positive stained cells (grey filled) and unstained cells (unfilled). Pooled data of the percentages of cells positive for CD11c (d), CD40 (g), CD86 (j) and CD83 (m). Surface expression levels were measured and shown as MFIs of CD11c (e), CD40 (h), CD86 (k) and CD83 (n) in young (n = 14) versus elderly (n = 25) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

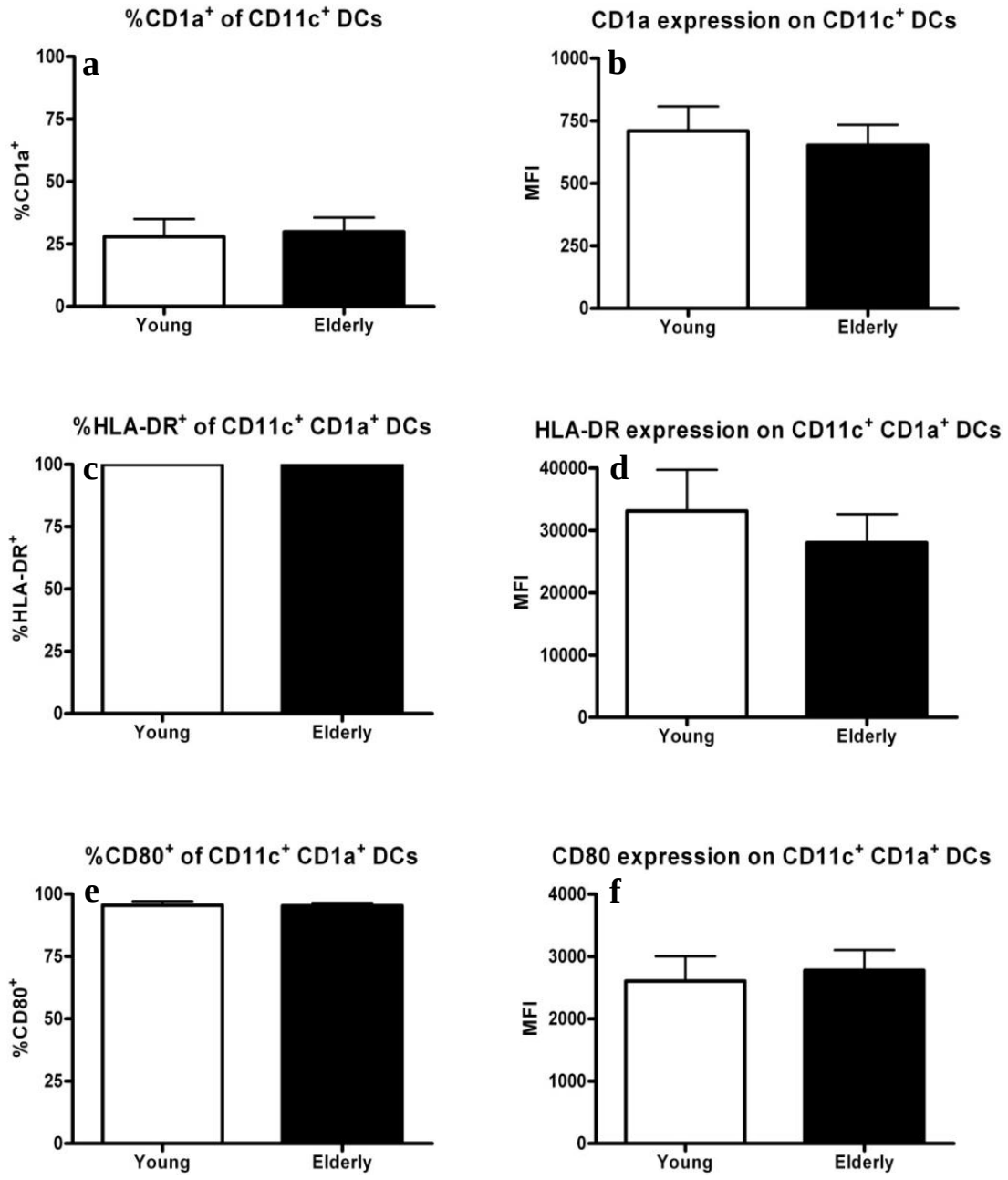

Figure 3.17: CD1a, HLA-DR and CD80 on LPS/IFNγ stimulated CD11c$^+$ MoDCs is not affected by age

Immature MoDCs generated from young and elderly volunteers were stimulated with LPS and IFNγ and cell surface molecules analysed by flow cytometry after gating on CD11c$^+$ cells. CD11c$^+$ DCs were further gated on CD1a$^+$ cells (a and b). CD11c$^+$CD1a$^+$ DCs were analysed for expression of HLA-DR (c and d) and CD80 (e and f). Pooled data from young (n = 14) versus elderly (n = 25) volunteers and is shown as mean ± SEM.

for HLA-DR and CD80 expression. With no age-related differences seen for either the percentage of cells positive for CD80 (Figure 3.17e; p = 0.78) or HLA-DR (Figure 3.17c; p = 0.96) or the expression levels of CD80 (Figure 3.17f; p = 0.92 and HLA-DR (Figure 3.17d; p = 0.37).

3.2.7 LPS/IFNγ stimulation does not fully rescue elderly-derived MoDCs from maturation paralysis

LPS/IFNγ stimulated MoDCs from 37 healthy volunteers were incubated with DQ-OVA and cells analysed by flow cytometry. Greater than 80% of young-derived LPS/IFNγ stimulated MoDCs lost their antigen processing ability (Figure 3.18a). In contrast, only 50% of elderly-derived LPS/IFNγ stimulated MoDCs demonstrated maturation by loss of antigen processing ability. The remaining 50% of MoDCs showed a down-regulated ability to process $FITC^{+}$DQ-OVA indicating only partial maturation (Figure 3.18b).

3.2.8 LPS/IFNγ MoDCs activated show an increased trend to induce CD8 proliferation with age

*LPS/IFN*γ activated MoDCs were incubated with CFSE labelled lymphocytes from the universal donor. No age-related differences were observed for CD4 T cell proliferation (Figure 3.18c). MoDCs from elderly volunteers showed a trend towards increased CD8 T cell proliferation at low DC:T cell concentrations relative to young volunteers (Figure 3.18d).

3.2.9 Age does not affect cytokine secretion in MoDCs activated by LPS/IFNγ

Culture media from LPS/IFNγ-activated MoDCs was collected and analysed for cytokine content by cytometric bead array. No difference was observed for the secretion of either VEGF (Figure 3.19c; p = 0.57) or IFNγ (Figure 3.19e; p = 0.49) by MoDCs from either elderly or young individuals. An age-related trend towards increased TNF (Figure 3.19a; p = 0.57), IL-10 (Figure 3.19b; p = 0.30) and IL-12p70 (Figure 3.19d; p = 0.27) secretion by MoDCs was observed.

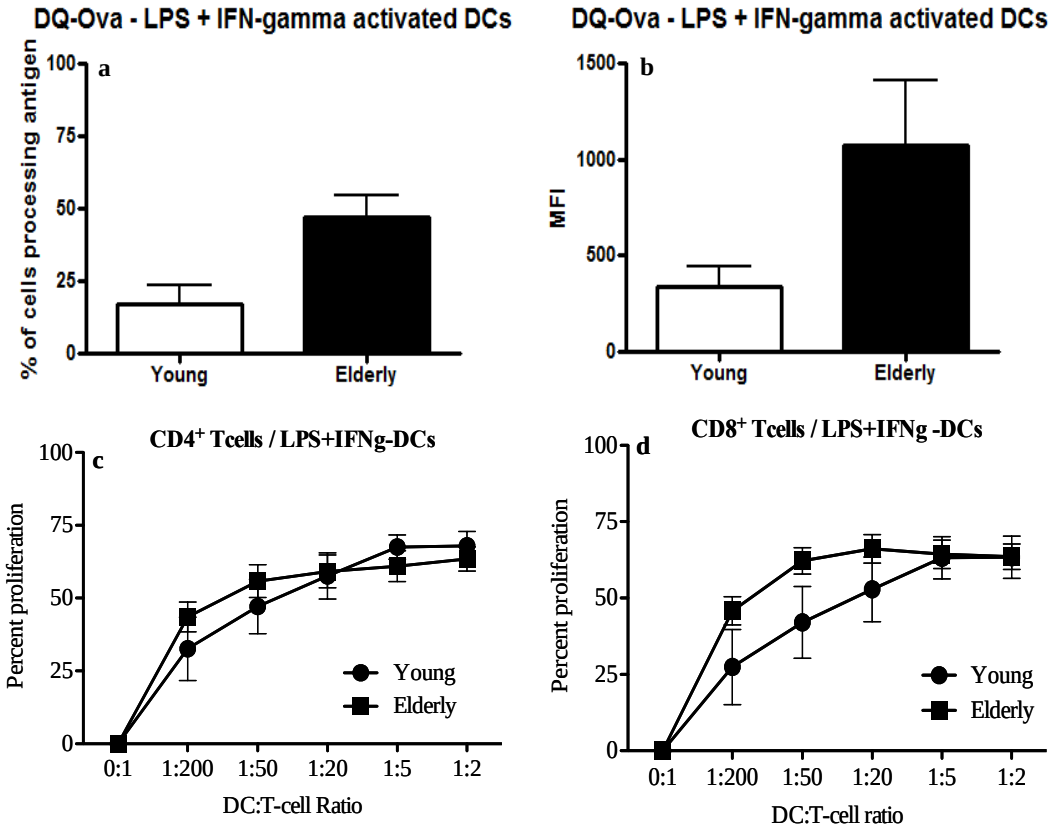

Figure 3.18: LPS+IFNγ elderly-MoDCs partially maintain their capacity to process antigen yet induce T cell proliferation

MoDCs from healthy young and elderly individuals previously stimulated with LPS/IFNγ were incubated for 1 hour with FITC-DQ-Ovalbumin (DQ-OVA). The capacity to process antigen was measured by flow cytometric analysis. Pooled data of the percentage of DCs still processing antigen (a) and MFIs indicating relative antigen processing capacity (b) is from n = 12 young and n = 25 elderly volunteers. LPS/IFNγ activated MoDCs were also co-cultured with allogeniec CFSE labelled lymphocytes for 7 days. Cells were collected, stained for CD4 and CD8 expression and analysed by flow cytometry. Pooled percentage proliferation was plotted against DC: T-cell ratio for young (n = 6) versus elderly (n = 12) MoDCs. Pooled data is shown as mean ± SEM. All P-values were determined using the two-tailed Mann-Whitney test. *$p < 0.05$

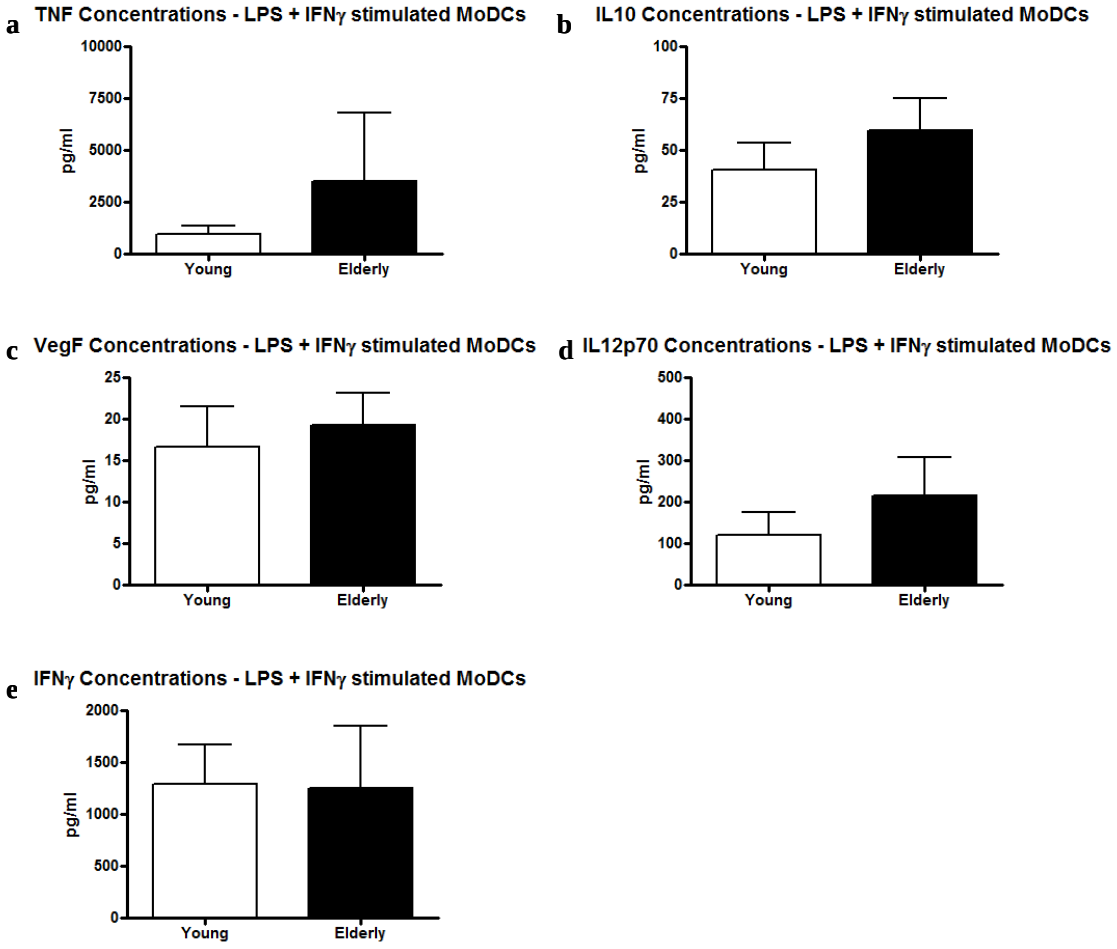

Figure 3.19: Age does not affect cytokine secretion in MoDCs activated by LPS + IFNγ

MoDCs from healthy young and elderly volunteers were stimulated with both LPS and IFNγ for 48 hours. Culture media was collected and analysed by cytometric bead array for the presence of cytokines. Cytokine concentration was determined by measuring the MFI of the corresponding beads as per Figure 10. Pooled data for the concentration of TNF (a), IL-10 (b), VEGF (c), IL-12p70 (d) and IFNγ (e) present in culture media containing young (n = 12) and elderly (n = 14) MoDCs. Pooled data is shown as mean ± SEM.

3.3 Discussion

Dendritic cells play an essential role in the initiation and control of immune responses (Banchereau et al., 2000). Several studies have investigated the effect of aging on the immune system. These studies show that whilst the bone marrow of elderly hosts produces more haemopoetic stem cells (HSCs), these cells have lower activity and tend to differentiate into macrophages rather than lymphocytes (Beerman et al., 2010, de Haan and Van Zant, 1999). Furthermore, whilst there is an increase in memory T cells, there is a decrease in naive T cells (Fahey et al., 2000, Yuan et al., 2005). Studies have also identified a decrease in antigen-specific T cells being produced (Naylor et al., 2005, elRefaei et al., 2001). As DCs regulate the induction and maintenance of antigen-specific T cells, any change in DC function could contribute to the T cell dysfunction seen in the elderly (Uyemura et al., 2002). There is only limited published data investigating the effect of aging on human dendritic cells, much of which is contradictory. The aim of these experiments was to identify any age related changes in dendritic cells in order to establish a suitable control population for comparison to mesothelioma patients in the following chapters.

Data from this study is in agreement with others showing that pDC numbers significantly decline with age (Jing et al., 2009, Orsini et al., 2012, Perez-Cabezas et al., 2007, Shodell and Siegal, 2002); this level of agreement may be partly due to examination of the same surface molecules, i.e. CD303 or CD123. The decreased pDC levels may explain why elderly individuals show an increased susceptibility to infections (Pawelec et al., 2002, Stout-Delgado et al., 2008, Tassone et al., 2010).

The use of surface markers to identify myeloid DC subsets has not been so consistent. These markers include CD1c (Perez-Cabezas et al., 2007) which allows for detection of the mDC1 subset, whilst ignoring the smaller but potentially more powerful mDC2 population. Using CD33 (Orsini et al., 2012) and CD11c (Jing et al., 2009, Della Bella et al., 2007) groups all myeloid DC subsets including mDC1s, mDC2s and MoDCs into a single population. This study is the first to examine the effect of aging on three separate blood dendritic cell subsets pDCs, mDC1s and mDC2s simultaneously. The results are

in agreement with others who did not observe age-related changes in myeloid DC subpopulation numbers (Jing et al., 2009, Orsini et al., 2012, Perez-Cabezas et al., 2007).

The observations described above contrast to Della Bella et al (2007) who reported a decrease in myeloid DCs due to age and no change in pDC numbers. There are two possible reasons for the variability of the results. Firstly, only Della Bella rigorously adhered to the SENIEUR protocol when selecting volunteers. The SENIEUR protocol is a more rigid selection criteria which apart from using clinical and pharmaceutical criteria, also excludes serum, haematological and urinalysis observations. The second reason could be variation due to regional differences.

The low numbers of circulating blood DCs makes it challenging to measure functional changes. Use of in vitro generated MoDCs, which behave in a similar manner to blood DCs (Ardavin et al., 2001, Sallusto and Lanzavecchia, 1994) overcomes this difficulty. Several studies, including this one, agree that monocytes from elderly subjects readily differentiate into phenotypically classical MoDCs. Whilst this study observed no significant difference in expression of key surface molecules, it did observe a decreased trend due to age for these molecules. Whilst previous studies reported no difference in expression of key molecules, close examination of their data shows that most molecules examined demonstrated a decreased trend with age (Ciaramella et al., 2011, Lung et al., 2000, Steger et al., 1996). In contrast, Agrawal et al (2007) observed an increase trend in CD86 expression due to age. It should be noted that the preparation of human MoDCs used by Agrawal differed from this study and previous studies in that it changed 50% of the culture media every two days during MoDC differentiation. It is possible that this could have altered the final population of MoDCs present to sufficiently change the measured CD86 expression.

This study investigated whether the function of MoDCs changed with aging. An important function of immature DCs is their ability to take up and process antigen in preparation for presentation to T cells; the latter occurs after DCs have matured. Others

have reported that elderly MoDCs demonstrate a decreased ability to take up antigen (Agrawal et al., 2007) which the authors believed to be receptor independent. This study showed that age did not impact on the percentage of immature MoDCs that could process antigen or the levels at which they processed antigen. Taken together, these data suggest that once elderly-derived DCs have taken up antigen their ability to process it is not impaired.

The present study also examined the capacity of immature MoDCs to present antigen to T cells. As expected, immature DCs are not as effective as mature DCs in presenting antigen. The results from this study showed that the ability of immature MoDCs to present antigen to $CD4^+$ and $CD8^+$ T cells was not significantly different between young and aging hosts. These data are similar to those from Steger et al (1996). Interestingly, immature MoDCs from elderly volunteers appeared to be slightly better at inducing T cell proliferation than those from younger volunteers, which may indicate partial maturation in the absence of a stimulus.

This study also compared MoDC responses to maturational stimuli. Several age-related changes were seen when immature MoDCs were activated with LPS, IFNγ or LPS plus IFNγ. The use of LPS alone induced lower expression levels for all surface markers examined, with a significant decrease in CD83 in elderly-derived DCs. CD83 is a marker of maturation thus, elderly-derived MoDCs may be impaired in their ability to fully mature. When MoDCs were activated with IFNγ decreased expression levels were seen in elderly-derived MoDCs for most surface markers, with the exception of increased levels of the co-stimulatory molecules CD80 and CD86. These data imply that elderly MoDCs respond more appropriately to IFNγ rather than to LPS in terms of phenotypic changes.

When MoDCs were activated with the combination of LPS and IFNγ only slight age-related phenotypic differences were observed. It is possible that the combination of the two stimuli generated a sufficiently strong maturation signal that overcame phenotypic defects in the elderly MoDC response to LPS alone. There is limited published data on

the effect of maturation stimuli on young-derived versus elderly-derived MoDCs. The two studies investigated the effect of age on LPS activated MoDCs observed no difference in the resulting phenotype (Agrawal et al., 2007, Ciaramella et al., 2011). A possible reason for the difference between the published studies and this study is the degree of activation induced in the MoDCs. In the studies by Agrawal and Ciaramella, only a slight increase in surface markers was observed following LPS activation. In contrast, this study observed much stronger up-regulation of surface markers. The published studies activated immature MoDCs with either 100 ng/ml or 200 ng/ml LPS, whilst this study activated with a significantly higher concentration, i.e. 10 μg/ml LPS. The smaller up-regulation of surface markers seen in the two published studies could be masking differences between the two age groups.

This is the first study to investigate the effect of aging on antigen processing ability following MoDC stimulation. As expected, 80% of young healthy MoDCs lost their antigen processing ability as they matured in response to LPS stimulation. However, a lower percentage (55%) of LPS-matured elderly MoDCs lost their antigen processing ability. Nonetheless, the processing capacity of the remaining 45% of cells was greatly reduced implying that the normal maturation process had been at least partially induced in elderly-derived MoDCs by LPS stimulation.

Significantly less elderly-derived MoDCs lost their ability to process antigen following stimulation with IFNγ. Indeed, the processing capacity was significantly higher in elderly IFNγ stimulated MoDCs compared with their younger counterparts. These data suggest an age-related defect in response to IFNγ. When LPS was combined with IFNγ to stimulate MoDCs, 80% of young-derived MoDCs compared to 55% of elderly-derived MoDCs responded appropriately by completely down-regulating their ability to process antigen. These data are similar to the LPS-only responses suggesting that the LPS/IFNγ combination did little to overcome the defect in response to IFNγ. This was further emphasized by the responses of the remaining 20% of young versus 45% of elderly-derived MoDCs to the LPS/IFNγ combination; only the younger MoDCs reduced their antigen processing capacity. Taken together, these data show that whilst

MoDCs from younger subjects respond appropriately to maturation stimuli, MoDCs from elderly subjects achieve a state of semi-maturation.

Despite defects seen in specific components of MoDC maturation in elderly hosts the results here show that regardless of the stimuli used elderly mature MoDCs demonstrated an improved capacity to present antigen at sufficient levels to induce $CD4^+$ and $CD8^+$ T cell proliferation. Indeed, elderly LPS-stimulated MoDCs showed a significantly greater capacity than their younger counterparts to induce $CD8^+$ T cell proliferation. These data are different to those from Steger et al (1996), who did not observe any differences in $CD4^+$ T cell proliferation when tetanus toxoid (TT) pulsed-DCs from young versus elderly donors were co-cultured with TT-specific T cells; however, their sample numbers were very low and the results of two elderly versus two young volunteers were compared which may be insufficient. The use of different antigens may also contribute to the observed differences, this study used alloantigen.

Following activation, MoDCs respond by secreting cytokines to induce TH_1/TH_2 polarization (Shortman and Liu, 2002). This study observed only minor differences with the majority of cytokines trending to increase with age when LPS was used to stimulate MoDCs. The exception was IFNγ which trended to decrease with age. Previous studies investigating cytokine secretion by MoDCs following stimulation have shown a wide variation in results. Ciaramella et al (2011), observed that following 48 hours of LPS stimulation, there was a significant decrease in IL-10 secretion, but no change in IL-12 secretion or TNFα secretion due to age. Alternatively, Agrawal et al (2007), observed a significant increase in TNFα secretion, with no differences in IL-12p70 or IL-10 secretion due to age. When Agrawal et al stimulated MoDCs with ssRNA they again saw a significant increase in TNFα and no difference in IL-10 (they did not report on IL-12p70 secretion). A study by Saurwein-Teissl et al (1998), observed that when MoDCs were stimulated with inactivated influenza virus, there was a significant increase in both IL-12 and TNFα irrespective of age. The variation observed between this study and previous studies could be due to lack of a standard for stimulating MoDCs. Each study

either used a different concentration or exposure time of LPS, or a different stimulus completely (e.g. LPS versus IFN versus ssRNA versus influenza virus).

In conclusion, this study shows that elderly individuals have decreased numbers of pDCs which may account for increased susceptibility to viral infection. Whilst MoDCs could be generated from blood monocyte precursors irrespective of age, several age-related differences were revealed after maturation using LPS and/or IFNγ stimuli. In particular, the antigen processing capacity normally only seen in immature DCs was maintained in IFNγ-stimulated elderly-derived MoDCs; these data imply a defect in the normal maturation process in aging hosts. Despite these defects, elderly-derived mature MoDCs demonstrated an improved capacity to present antigen at sufficient levels to induce $CD4^+$ and $CD8^+$ T cell proliferation. These data imply that elderly-derived DCs maintain key functional capacities that protect the host and that any defects seen may be targeted to improve this protection. The latter is the subject of future studies. This work provided essential background data prior to the selection of study comparator groups for the remainder of this thesis. Mesothelioma and other cancers have a higher incidence with advancing age. These results confirmed the necessity to use age-matched control subjects when studying DC function in subjects with cancer and other acute or chronic illness.

4 THE EFFECT OF MESOTHELIOMA ON DC SUBSETS

4.1 Introduction

As described in the previous chapter, dendritic cells (DC) are key antigen presenting cells that take up, process and present antigens to naïve T cells to induce antigen-specific immune responses (Banchereau et al., 2000, Banchereau and Steinman, 1998). DCs consist of a number of different but functionally related subsets. The studies in chapter 3 examined differences between blood pDCs, MDC-1, MDC-2 and MoDC subsets taken from healthy young versus healthy elderly donors. The data showed several age-related differences in DC subsets. Whilst elderly individuals had normal numbers of circulating mDCs, the number of circulating pDCs was significantly decreased. Elderly-derived monocytes readily differentiated into MoDCs, but did not upregulate costimulatory molecule expression to the same levels as their younger counterparts. Elderly-derived MoDCs also exhibited partial maturation paralysis following stimulation, in particular after exposure to IFNγ; specifically their ability to process antigen was not completely downregulated as seen in MoDCs from younger hosts. These data suggest that healthy elderly patients may have an impaired immune system that is independent of any disease state. These data also highlighted the necessity of using age-matched controls when investigating immune capacity in the setting of disease.

Mesothelioma is an aggressive cancer of the mesothelial lining around organs, and most commonly occurs in the pleural cavity. It is also a cancer that generally emerges in aging populations (Leigh and Driscoll, 2003) as it has a long latency period between asbestos exposure and detectable disease (Lanphear and Buncher, 1992). Thus, as mentioned above, this is a patient group that may already have a compromised immune system due to age-related defects, which may be further affected by the presence of mesothelioma tumour. To date, there are no published data on the numbers and function of DCs in people with mesothelioma. Yet DCs are critical for the generation of effective cytotoxic T lymphocyte (CTL) responses, a cell type that can lyse tumour cells and mediate tumour regression. Moreover, studies in other cancers have shown that DC numbers and function are often compromised in tumour-bearing hosts, and murine studies show that

the impact that the presence of tumour has on DC function, worsens with age (Grolleau-Julius et al., 2009, Grolleau-Julius et al., 2008, Guo et al., 2014).

Studies in some human cancers, including squamous cell carcinoma of the head and neck, prostate cancer, pancreatic cancer, breast cancer, Kaposi sarcoma and multiple myeloma have identified significant decreases in the numbers of circulating mDCs (Hoffmann et al., 2002, Ma et al., 2009, Sakakura et al., 2006), pDCs (Sciarra et al., 2007), or both (Della Bella et al., 2006, Harrison et al., 2008, Pinzon-Charry et al., 2007, Tjomsland et al., 2010). Several studies have investigated whether decreased blood DC numbers are due to a tumour-induced defect during the process of differentiation from precursor cells into DCs. DCs extracted from tumours have been shown to express an immature phenotype that is associated with reduced capacity to induce T cell proliferation (Troy et al., 1998a, Troy et al., 1998b). Furthermore, both circulating blood DCs and MoDCs generated from patients with breast cancer, hepatocellular carcinoma and squamous cell carcinomas of the head and neck are reported to express significantly lower levels of CD80, CD86 and/or HLA-DR (Ma et al., 2009, Ninomiya et al., 1999, Pinzon-Charry et al., 2007, Sakakura et al., 2006) relative to DCs from healthy volunteers. These data imply an inability to appropriately activate T cells. Mouse models of mesothelioma have shown that tumour antigen presentation to CTLs does occur in draining lymph nodes (Jackaman et al., 2012b, McDonnell et al., 2010) however, the role of DCs in this process has yet to be clarified.

The limited studies investigating the effect of cancer on DC responses to stimuli have used different DC types and stimuli generating contradictory results. Harrison et al. (2008), observed increased levels of surface CD83 and CD86 following ex vivo TNF-α and PGE_2 stimulation in blood DCs from multiple myeloma patients relative to age matched controls. Pinzon-Charry et al. (2007), observed decreased CD80 and HLA-DR expression in blood DCs from breast cancer patients compared to age matched controls regardless of whether the ex vivo stimulus was a cytokine cocktail (IL-1β, IL-6, TNF-α and PGE_2), poly I:C or CD40L. Similarly, Onishi et al. (2002), observed decreased levels of CD80 on MoDCs derived from patients with various advanced cancers

(stomach, colon, ovary, lung and breast) after exposure to monocyte conditioned media containing IL-1β, GM-CSF, TNF-α and IFNγ.

A few studies have investigated whether antigen uptake is impaired in DCs from patients with cancer. Pinzon-Charry et al. (2007) observed that blood DCs from people with breast cancer had a decreased ability to take up antigen relative to controls. In contrast, Ninomiya et al. (1999) observed that MoDCs from people with hepatocellular carcinoma had an increased ability to take up antigen. Interestingly, the study by Pinzon-Charry et al. was predominantly early stage patients, whilst those observed by Ninomiya et al. were predominantly late stage. This could indicate an early effect of cancer on DCs to aid in tumour escape, or merely a difference in tumour escape mechanisms between cancers. Neither study investigated whether antigen-processing ability was affected.

A key role of dendritic cells is the presentation of antigen to T cells. Pinzon-Charry et al. (2007) observed that the capacity of blood DCs to induce T cell proliferation became significantly decreased in patients with early stage breast cancer, and further decreased in advanced stage breast cancer. Similarly, Ma et al. (2009) observed that TNF-α stimulated MoDCs from patients with laryngeal squamous cell carcinoma also had a decreased ability to drive T cell proliferative compared to healthy controls.

The studies presented in this chapter aimed to determine the effect of mesothelioma on the number of pDCs, mDC1s and mDC2s in the peripheral blood of patients compared to healthy aged matched controls. The study also assessed the capacity for monocytes from both cohorts to differentiate into MoDCs, as well as their ability to process and present antigen and respond to both microbial and cytokine stimuli.

4.2 Results

4.2.1 Characteristics of study volunteers

A total of 48 volunteers with mesothelioma were recruited for this study by three clinicians, Professor Anna Nowak, Professor Bill Musk and Dr John Alvarez, with three additional patients recruited through radio advertising. Patients were excluded from the study if they had undergone active anticancer treatment (chemotherapy, radiotherapy, surgery) in the previous 9 months. Greater than 90% of patients included in the study were newly diagnosed. As described in chapter 3 all healthy volunteers were recruited by (i) radio advertising, (ii) print advertising in an elderly demographic newspaper, (iii) poster advertising at Curtin University, and (iv) recruitment from laboratory volunteers. Healthy volunteers were excluded from the study if they currently had cancer, autoimmune disorders or other severe immune disorders. For the purposes of this study, volunteers were dichotomised into two groups: mesothelioma patients were aged between 47 and 84 years of age whilst healthy age matched controls were aged between 48 and 84 years of age (Table 4.1). The minimum age limit chosen in chapter 3 was lowered in this chapter to match the age range of the mesothelioma patients used in the study. Signed consent was obtained prior to blood collection. Health status was determined by collecting data on current and past medical conditions of the volunteers and their biological family using a survey form. Approval for this study was given by the Human Ethics Committees for the Sir Charles Gairdner Hospital, Perth, Western Australia (#2008-041); the Mount Hospital, Perth, Western Australia (#EC50.1), and for Curtin University, Bentley, Western Australia (#HR68/2008).

Table 4.1: Characteristics of study volunteers

	Mesothelioma	**Age Matched**	**p Value**
Number of subjects:	48	40	
Age range in years:	47 - 84	48 - 84	
Mean age in years:	66.90	67.50	0.7092
Standard deviation of age:	8.365	8.277	
Female gender no. (%):	10 (20.8)	15 (37.5)	0.1723

4.2.2 People with mesothelioma have decreased numbers of blood DC subsets

The numbers of circulating DC subsets was analyzed from 48 mesothelioma patients and 36 age matched controls. Cell numbers per ml of blood were determined using a blood dendritic cell enumeration kit and flow cytometry as described in chapter 3, Figure 3.1.

As shown in Chapter 3, all blood DC subsets in healthy individuals showed an age-related decrease in numbers, with pDCs demonstrating a statistically significant decrease. All DC subset numbers in people with mesothelioma were even further reduced relative to the age-matched controls, and in the disease state, this appeared independent of age. All differences were statistically significant; i.e. mDC1s (Figure 4.1e: $p < 0.0001$), mDC2s (Figure 4.1f: $p < 0.0001$) and pDCs (Figure 4.1g: $p < 0.0001$). This data suggest that mesothelioma impairs either the generation of circulating DCs, or induces apoptosis in circulating DCs, beyond what was observed due to age alone.

4.2.3 Mesothelioma impairs the ability of monocytes to differentiate into $CD40^+$ immature MoDCs

As functional analysis of *ex vivo* blood DC subsets are difficult due to low numbers the next series of experiments involved the generation of MoDCs from monocyte precursors *in vitro*. Initially, monocytes from people with mesothelioma and healthy controls were exposed to GM-CSF and IL-4 (Figure 4.2a) and their ability to differentiate into immature MoDCs investigated by examining expression of key DC surface markers on gated $CD14^-$ cells (Figure 4.2b).

No differences were observed between people with mesothelioma and age-matched controls in the percentage of $CD14^+$ cells that differentiated into classical $CD14^-CD11c^+$ (Figure 4.2c) dendritic cells (Figure 4.2d; $p = 0.27$). Whist a trend towards lower CD11c surface expression levels (as assessed by MFI) was observed in people with mesothelioma, wide individual variation in healthy controls meant that the difference did not reach statistical significance (Figure 4.2e; $p = 0.43$).

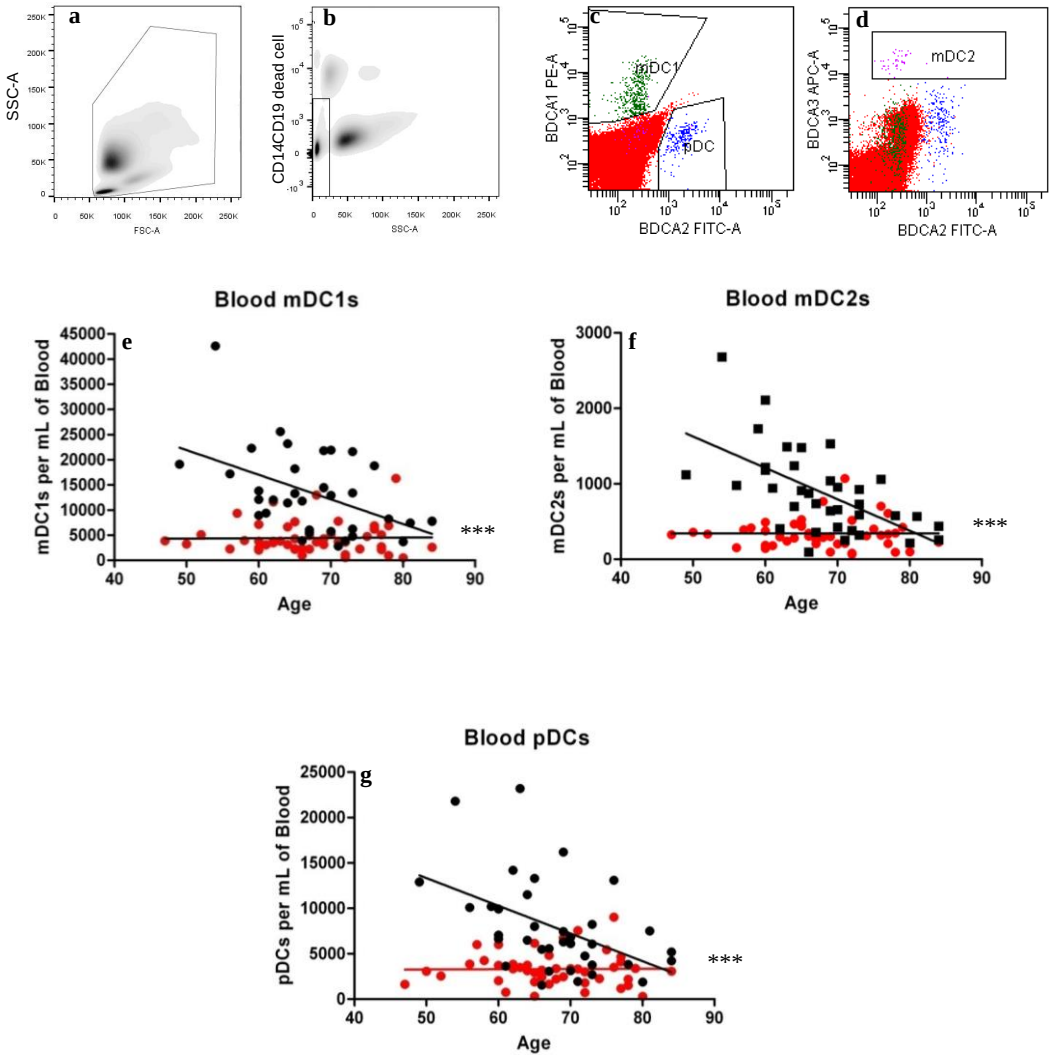

Figure 4.1: People with mesothelioma have decreased numbers of blood DC subsets

Whole blood was stained for blood DC subpopulations and analysed by flow cytometry. Representative dot plot (a) showing gating of leukocytes by size (FSC) and granularity (SSC). $CD14^+$ monocytes, granulocytes and $CD19^+$ B-cells as well as dead cells were further excluded by gating (b). Blood DC subpopulations were identified by high expression of BDCA-1 (c: mDC1), BDCA-3 (d: mDC2) and BDCA2 (c: pDC). The absolute number of circulating, myeloid dendritic cells (mDC1: e, and mDC2: f) and plasmacytoid dendritic cells (pDC; g) measured as the number of DCs per ml of blood plotted against age. Absolute counts were determined by multiplying the number of DCs in the leukocyte gate by the number of PBMCs determined by counting on a haemocytometer. Each dot (mesothelioma: red dots, age-matched: black dots) represents an individual volunteer (mesothelioma: n = 48, age matched: n = 36), with the lines representing linear regression. P-values were determined using the two-tailed Mann-Whitney test. ***$p < 0.0001$.

As mentioned in chapter 3, CD40 (Figure 4.2f) and CD86 (Figure 4.2i) are important co-stimulatory molecules and CD83 (Figure 4.2j) is a key maturation marker on DCs. No differences were seen in the percentage of cells expressing CD86 (Figure 4.2j: $p = 0.51$). A quantitatively small and non-significant trend towards a decrease in the percentage of cells expressing CD40 (Figure 4.2g: $p = 0.14$) and CD83 (Figure 4.2m: $p = 0.06$) was observed in people with mesothelioma. A trend for decreased surface expression levels of CD86 (Figure 4.2k: $p = 0.54$) and CD83 (Figure 4.2n: $p = 0.15$) was observed in people with mesothelioma. The differences in CD83 expression again did not reach significance due to the wide individual variation seen in the healthy controls. However, a significant decrease in CD40 surface expression was observed (Figure 4.2h: $p = 0.034$); these data imply an impairment during differentiation into iMoDCs which could affect the downstream polarization of CD40 ligand$(L)^+$ $CD4^+$ T cells into Th1 cells.

$CD11c^+$ DCs can differentiate into $CD11c^+CD1a^+$ DCs or $CD11c^+CD1a^-$ DCs that play a pro-inflammatory or anti-inflammatory role respectively (Cernadas et al., 2009, Chang et al., 2000). Their presence in cancer patients may represent their immune status. Therefore, immature MoDCs were further gated as $CD11c^+$ cells (Figures 4.3a and 4.3b) then as $CD1a^+CD11c^+$ DCs (Figure 4.3c). No differences were observed between MoDCs from mesothelioma patients and controls in either the percentage of $CD11c^+$ cells expressing CD1a (Figure 4.3d: $p = 0.81$) or CD1a surface expression levels (Figure 4.3e: $p = 0.65$). $CD1a^+CD11c^+$ DCs were further examined for expression of HLA-DR (a MHC class II molecule involved in antigen presentation to $CD4^+$ T cells) and the co-stimulatory molecule CD80. Whilst no differences were seen for the percentage of cells expressing HLA-DR (Figure 4.3g: $p = 0.14$) or CD80 (Figure 4.3j: $p = 0.56$), trends were observed for decreased surface expression levels of HLA-DR (Figure 4.3h: $p = 0.057$) and CD80 (Figure 4.3k: $p = 0.12$). These data suggest that DCs from people with mesothelioma have the same capacity to develop into pro-inflammatory $CD11c^+CD1a^+$ DCs as healthy controls, although slightly lower HLA-DR and CD80 expression may impact on T cell activation.

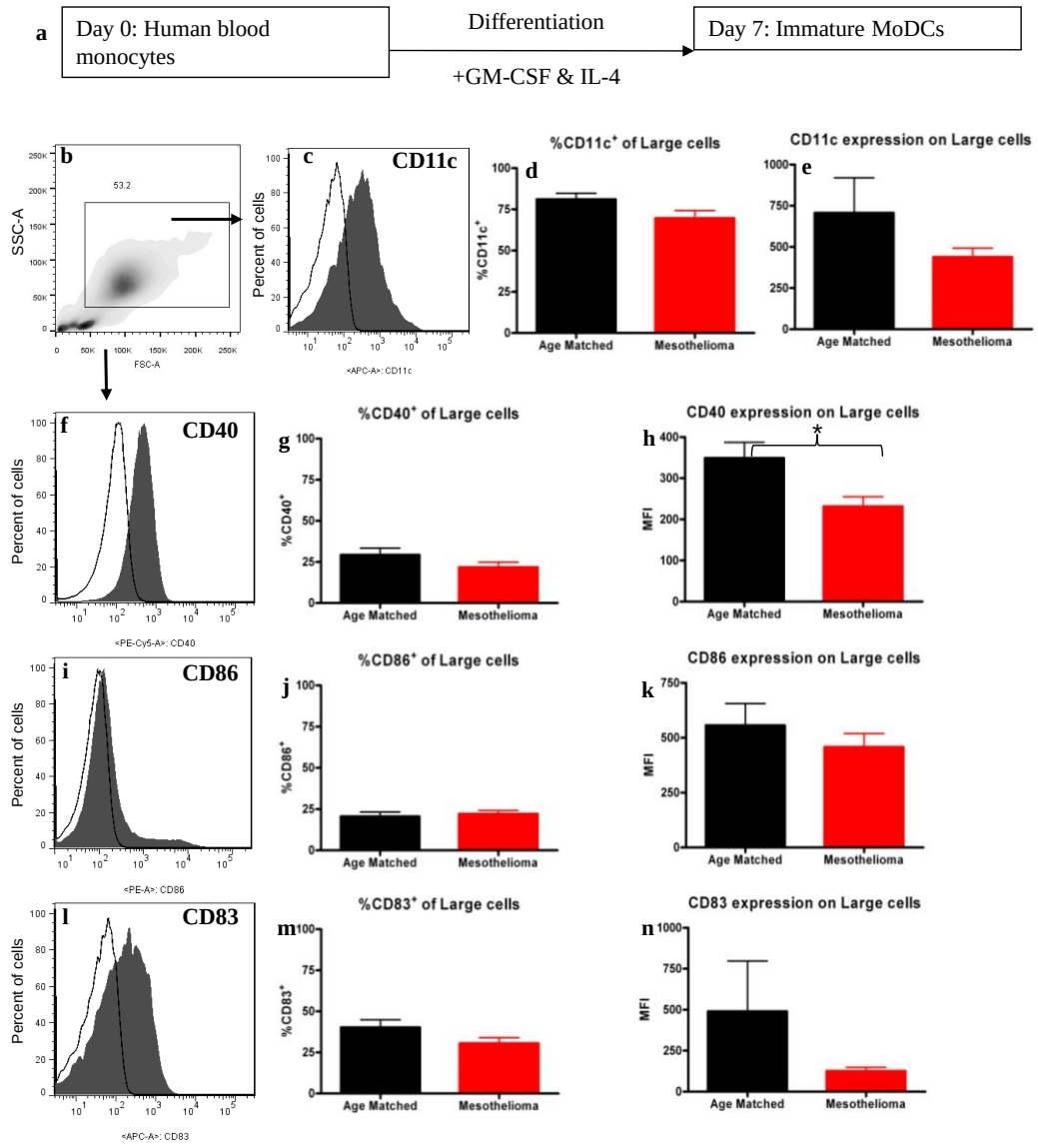

Figure 4.2: CD40 expression in mesothelioma iMoDCs is reduced compared to age matched iMoDCs

Human monocytes were differentiated into iMoDCs using GM-CSF and IL-4 (a). Immature MoDCs from mesothelioma patients and age matched volunteers were collected and cell surface molecules analysed by flow cytometry. Representative plot (b) showing gating of large cells which were analysed for expression of CD11c (c), CD40 (f), CD86 (i) and CD83 (l) with positive stained cells (grey filled) and unstained cells (unfilled). Pooled data of the percentages of cells positive for CD11c (d), CD40 (g), CD86 (j) and CD83 (m). Surface expression levels were measured as MFIs of CD11c (e), CD40 (h), CD86 (k) and CD83 (n) in mesothelioma patients (n = 46) versus age matched (n = 27) iMoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test. *$p < 0.05$

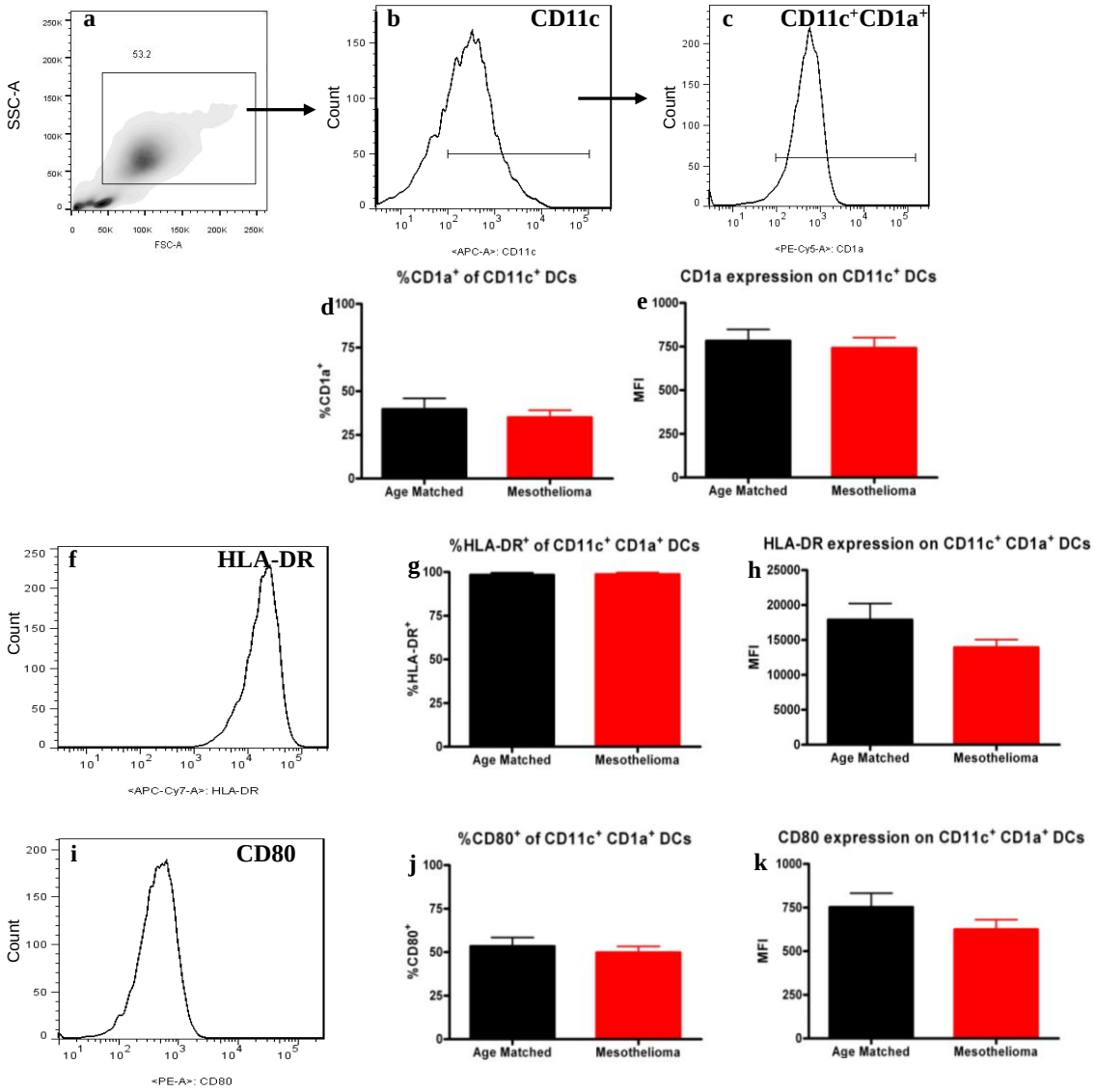

Figure 4.3: Mesothelioma induces a decreased trend in CD80 and HLA-DR expression in iMODCs

Immature MoDCs from mesothelioma patients and age matched volunteers were collected and cell surface molecules analysed by flow cytometry. Large cells (a) were identified and gated on $CD11c^+$ cells (b). $CD11c^+$ DCs were further analysed and gated on $CD1a^+$ cells (c). $CD11c^+CD1a^+$ DCs were analysed for expression of HLA-DR (f) and CD80 (i). Pooled data for the percentage of cells positive for CD1a (d), HLA-DR (g) and CD80 (j) and for cell surface expression levels (MFIs) of CD1a (e), HLA-DR (h) and CD80 (k) is from young (n = 46) versus elderly (n = 27) iMoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test.

4.2.4 Immature MoDCs from patients with mesothelioma cannot readily process antigen

The primary role of immature DCs is to take up and process antigen. The DQ-OVA assay was used to compare the antigen processing ability of MoDCs between 42 mesothelioma patients and 29 healthy volunteers. Antigen processing ability was measured as per chapter three, briefly iMoDCs were first gated for by size (Figure 4.4a) then for the degradation of FITC-labeled DQ-Ovalbumin, which indicates antigen processing (Figure 4.4b). Whilst no differences were observed for the percentage of MoDCs able to process antigen (Figure 4.4c: $p = 0.081$), MoDCs derived from people with mesothelioma demonstrated significantly lower levels of antigen processing (as measured using MFIs) relative to the healthy controls (Figure 4.4d: $p = 0.0035$). These data reveal an important defect in DCs from mesothelioma patients, i.e. their ability to process antigen is significantly reduced.

4.2.5 Mesothelioma does not interfere with the ability of iMoDCs to induce T cell proliferation

The primary function of mature DCs is to present processed antigen to naïve T cells in lymph nodes, whereas immature DCs are poor presenters of antigen. Immature MoDCs from 21 people with mesothelioma and 12 healthy age matched volunteers were co-cultured with CFSE-labeled allogeneic lymphocytes from a universal young healthy 34 year old male donor. Lymphocytes were first gated by size (Figure 4.5a) then by surface expression of CD4 and CD8 molecules (Figure 4.5b). Proliferation was identified by the decrease in CFSE staining intensity due to cell division. Proliferation was determined by the difference in $CFSE^{high}$ parent population (Figure 4.5c) compared with unstimulated lymphocytes. Both the mesothelioma patients and the healthy age-matched controls MoDCs demonstrated a similar ability to induce $CD4^+$ and $CD8^+$ T cell proliferation (Figures 4.5d and 4.5e). This data implies that the observed impairments in phenotype and antigen processing on mesothelioma patient iMoDCs do not impact on their ability to present antigen.

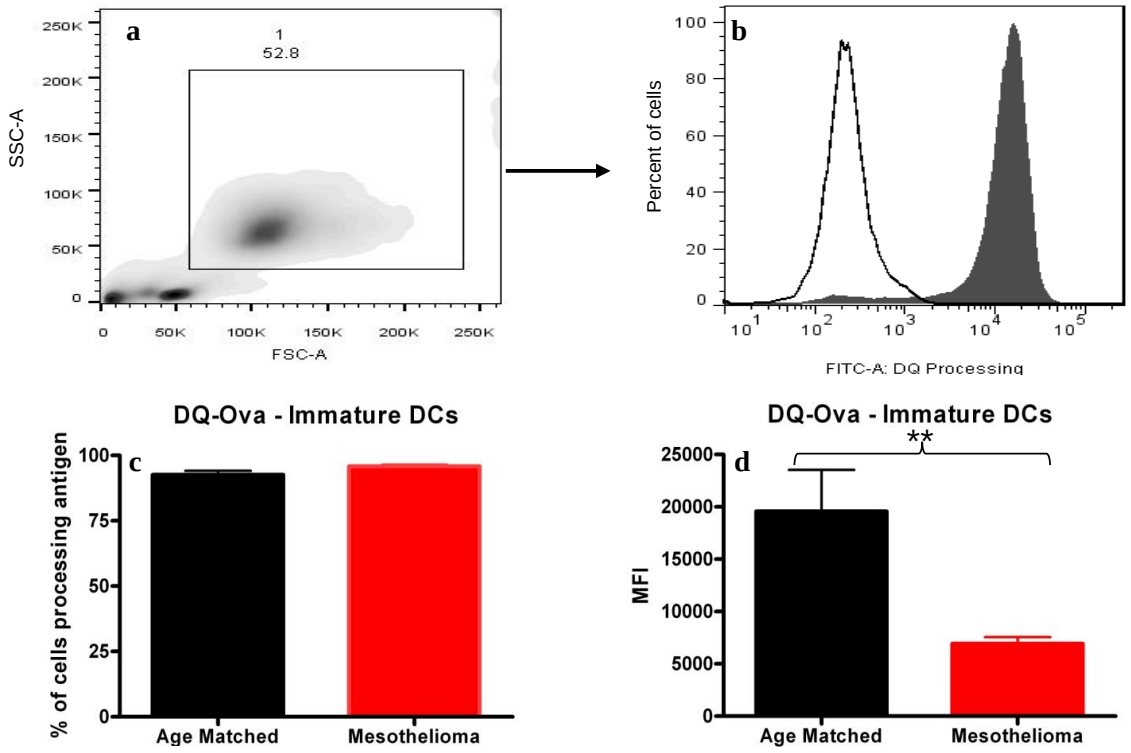

Figure 4.4: iMoDCs from mesothelioma patients have a reduced capacity to process antigen

Immature MoDCs from mesothelioma patients and age matched controls were incubated for 1 hour with FITC-labeled DQ-Ovalbumin (DQ-OVA). Representative dot plot (a) showing gating of MoDCs based on size (FSC) and granularity (SSC). The capacity to process antigen was determined by emission of a signal in the FITC channel (b) and measured by flow cytometry; grey histogram represents cells incubated with FITC-DQ-OVA, white histogram represents control cells that did not receive DQ-OVA. Pooled data (c) of % of DCs able to process antigen and (d) the mean fluorescent intensity (MFI) indicating relative antigen processing capacity of mesothelioma (n = 42) versus age matched (n = 29) iMoDCs. Pooled data is shown as mean ± SEM. **p < 0.01

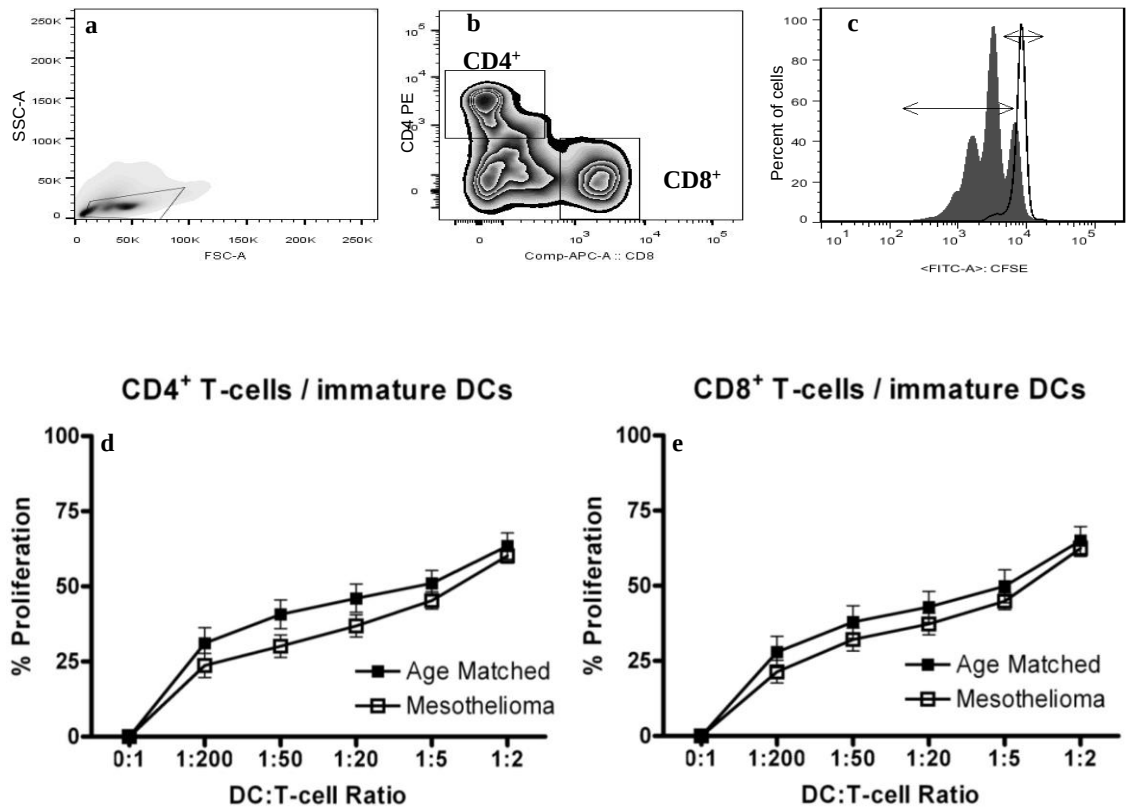

Figure 4.5: Mesothelioma does not modulate the ability of iMoDCs to induce lymphocyte proliferation

Immature MoDCs were co-cultured with allogeneic CFSE-labelled lymphocytes for 7 days. Cells were collected and stained for CD4 and CD8 expression and analysed by flow cytometry. Representative plot (a) showing gating of lymphocytes by size (FSC) and granularity (SSC). Lymphocytes were further gated as either $CD4^+$ or $CD8^+$ (b). The percentage of proliferating cells of the total gated population (longer arrow) was determined; the smaller arrow shows the non-proliferating parent peak. Pooled percentage proliferation was plotted against DC:T-cell ratio for $CD4^+$ (d) and $CD8^+$ (e) T-cells in age matched (n = 12) versus mesothelioma patients (n = 21) iMoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

4.2.6 LPS-stimulated MoDCs from patients with mesothelioma express lower levels of co-stimulatory molecules,

The capacity of appropriately matured DCs to present antigen is critical for the induction of T cell responses therefore, the next series of experiments examined DC responses to the microbial component lipopolysaccharide (LPS) and/or IFNγ. To investigate whether mesothelioma impairs LPS-driven maturation, iMoDCs from 46 patients with mesothelioma and 27 healthy age-matched volunteers were exposed to LPS for 48 hours (Figure 4.6a). DCs were gated by size and expression of CD11c, CD40, CD86 and CD83 investigated, as shown in Figure 4.2. No differences between iMoDCs from people with mesothelioma and controls were seen in the percentage of MoDCs expressing CD11c (Figure 4.6b: $p = 0.16$), CD40 (Figure 4.6d: $p = 0.28$), CD86 (Figure 4.6f: $p = 0.60$) or CD83 (Figure 4.6h: $p = 0.31$). Decreased surface expression levels were observed in MoDCs from patients with mesothelioma for CD11c (Figure 4.6c: $p = 0.18$), CD40 (Figure 4.6e: $p = 0.13$), CD86 (Figure 4.6g: $p = 0.088$) and CD83 (Figure 4.6i: $p = 0.13$) however, the differences relative to controls did not reach statistical significance.

LPS-matured MoDCs were also gated as $CD11c^+$ cells (as shown in Figure 4.3). No differences were observed in the percentage of $CD11c^+$ cells co-expressing CD1a (Figure 4.7a: $p = 0.87$), although there was a trend to decreased CD1a surface expression levels in people with mesothelioma (Figure 4.7b: $p = 0.51$). $CD1a^+CD11c^+$ DCs were then examined for expression of HLA-DR and CD80; no differences between patients and controls were noted in the percentage of DCs expressing HLA-DR (Figure 4.7c: $p = 0.24$) or CD80 (Figure 4.7e: $p = 0.97$), although again, a trend towards decreased expression levels of HLA-DR (Figure 4.7d: $p = 0.14$) and CD80 (Figure 4.7f: $p = 0.41$) was seen in the patient group.

4.2.7 LPS-matured MoDCs from patients with mesothelioma lose their capacity to process antigen

The next experiments further examined whether MoDCs from people with mesothelioma appropriately matured in response to LPS stimuli by losing their capacity

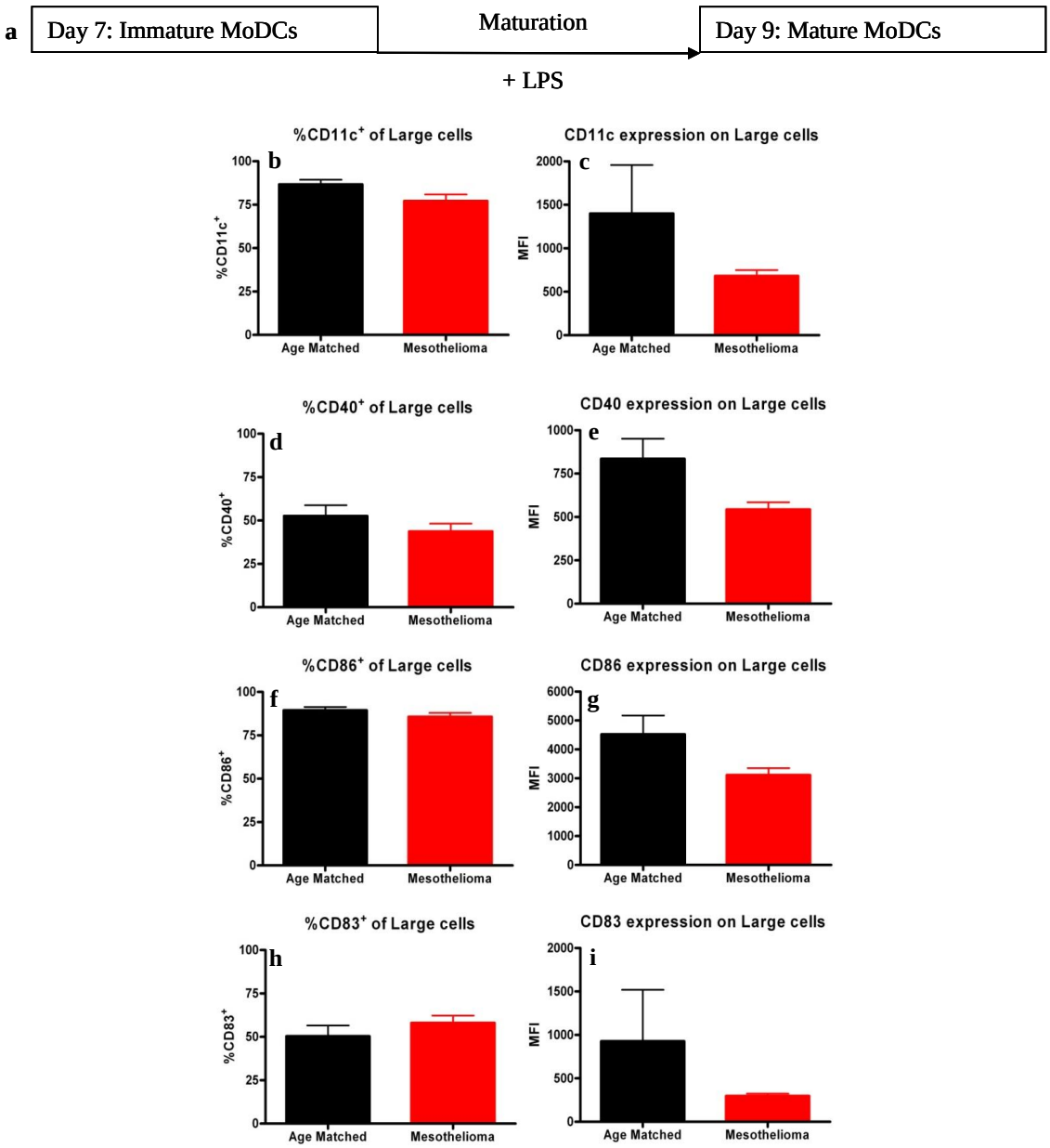

Figure 4.6: LPS-stimulated MoDCs from mesothelioma patients express lower levels of of CD11c, CD40, CD83 and CD86

Immature MoDCs generated from mesothelioma patients and age matched volunteers were stimulated with LPS (a) and cell surface molecules analysed by flow cytometry. Pooled data of the percentages of cells positive for CD11c (b), CD40 (d), CD86 (f) and CD83 (h). Surface expression levels were measured as MFIs of CD11c (c), CD40 (e), CD86 (g) and CD83 (i) in mesothelioma patients (n = 46) versus age matched (n = 27) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

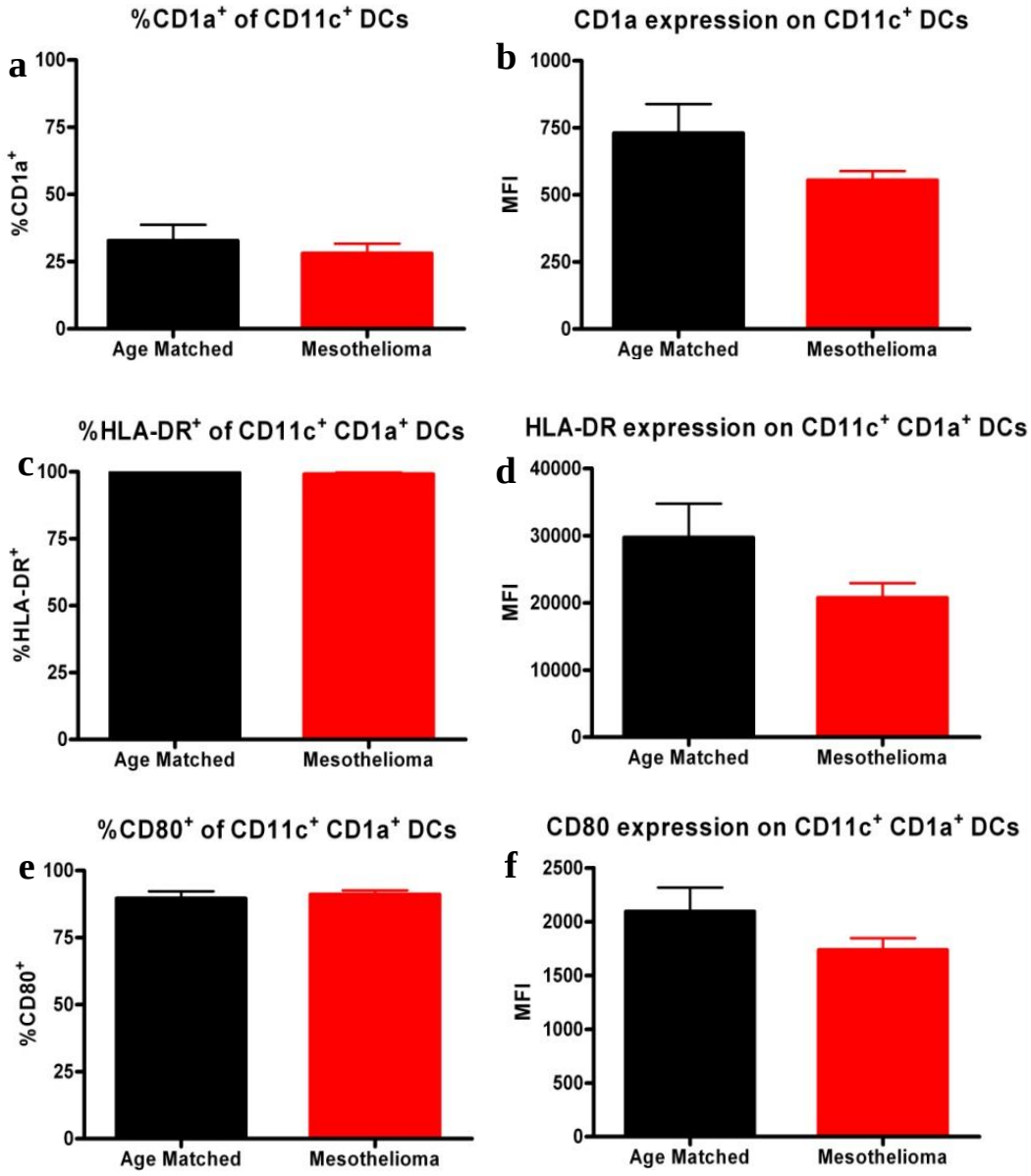

Figure 4.7: LPS-stimulated MoDCs from mesothelioma patients express lower levels of CD1a, HLA-DR and CD80

Immature MoDCs generated from mesothelioma patients and age matched volunteers were stimulated with LPS and cell surface molecules analysed by flow cytometry. $CD11c^+$ DCs were analysed for $CD1a^+$ expression (a,b). $CD11c^+CD1a^+$ DCs were analysed for expression of HLA-DR (c,d) and CD80 (e,f). Pooled data for the percentage of cells positive for CD1a (a), HLA-DR (c) and CD80 (e) and for cell surface expression levels (MFIs) of CD1a (b), HLA-DR (d) and CD80 (f) is from mesothelioma patients (n = 46) versus age matched (n = 27) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

to process antigen; measured using the DQ-OVA assay (described in Figure 4.4). Unlike DCs from young healthy donors (shown in chapter 3), the age-matched controls (> 47 years old) in this study did not fully lose their antigen processing ability after LPS stimulation. Indeed, > 45% of the age-matched LPS-matured control MoDCs retained their antigen processing ability (Figure 4.8c). These data imply that age-matched control MoDCs do not mature in response to LPS at equivalent levels to their younger counterparts (as discussed in chapter 3). In contrast, MoDCs from patients with mesothelioma showed significantly reduced percentages of cells that were processing the OVA antigen in association with significantly reduced levels of FITC-labeled DQ-OVA (Figures 4.8c and 4.8d). Whilst these data imply that MoDCs from patients have a greater maturation response to LPS than age-matched controls, the relative change from immature MoDCs for patients versus healthy age-matched controls was not significant (Figure 4.8e); i.e. the starting point for mesothelioma-derived iMoDCs to process antigen is already reduced relative to controls (refer to Figure 4.4d) and maturation with LPS leads to a further decrease in antigen processing ability.

4.2.8 LPS-matured mesothelioma-derived DCs maintain their ability to induce T cell proliferation

The ability of a DC to present antigen to T cells increases with maturation. Therefore LPS-matured MoDCs from 21 patients with mesothelioma and 12 age-matched healthy volunteers were also examined for their ability to induce proliferation of T cells from the universal donor. CD4 and CD8 T cells were identified by size and surface marker expression (as described in Figure 4.5). No differences were noted in either $CD4^+$ or $CD8^+$ T cell proliferation induced by MoDCs from patients compared to healthy age-matched controls (Figures 4.9a and 4.9b).

4.2.9 LPS-activated MoDCs from mesothelioma patients secrete increased TNF and IL-12p70

To determine if mesothelioma alters the secretion of cytokines following activation, culture media from mesothelioma patient and healthy age-matched control DCs was analysed for the secretion of TNF, IL-10, VEGF, IL-12p70 and IFNγ (as per Figures

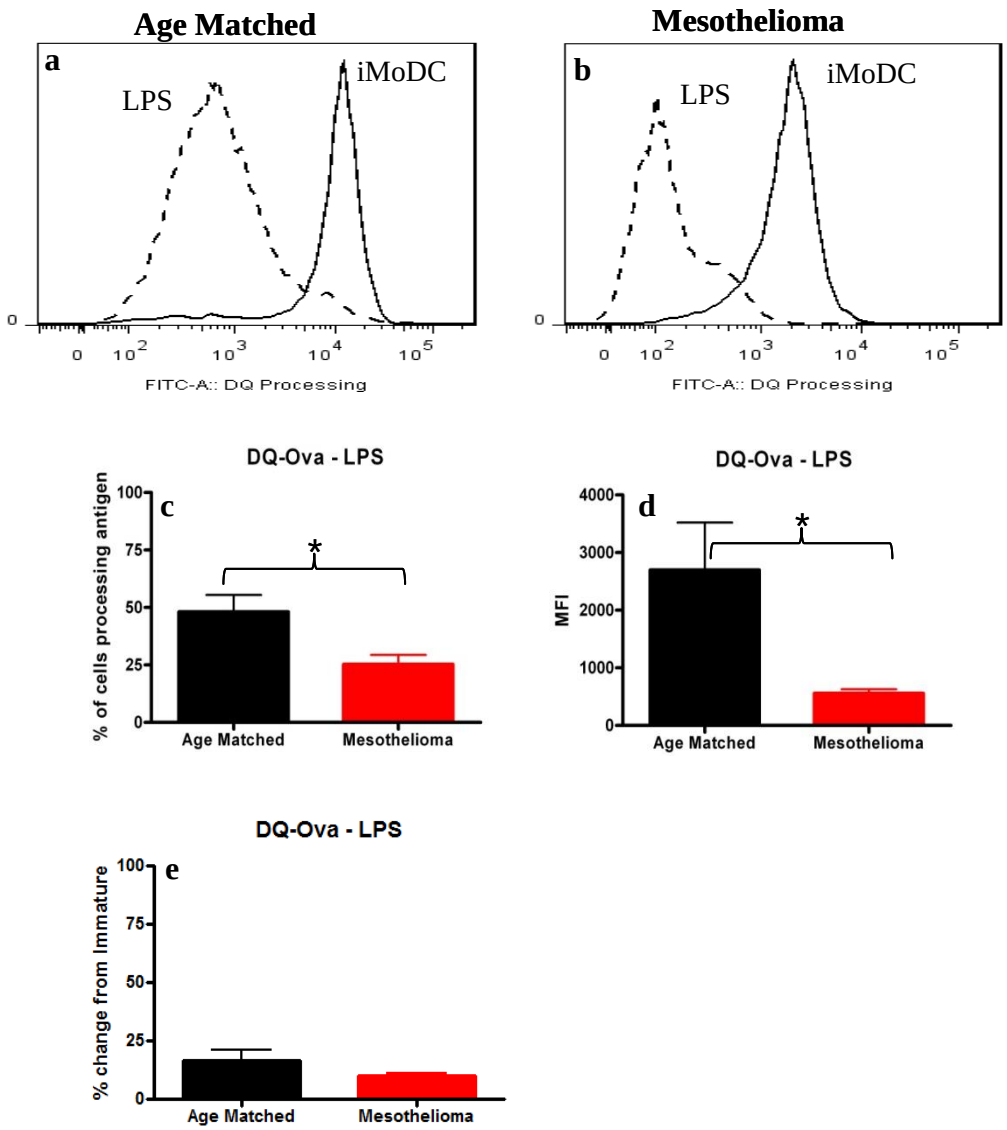

Figure 4.8: LPS-matured MoDCs from mesothelioma patients lose their capacity to process antigen

MoDCs from mesothelioma patients and healthy age-matched volunteers previously stimulated with LPS were incubated for 1 hour with FITC-DQ-Ovalbumin (DQ-OVA) as per Figure 4.4. The capacity to process antigen was measured by flow cytometric analysis. Representative histograms from mesothelioma patients (a) versus age-matched (b) derived iMoDCs and LPS-stimulated MoDCs. Pooled data of MFIs indicating relative antigen processing capacity (c), the percentage of DCs still processing antigen (d) and the percentage change from immature for LPS-treated age matched (n = 29) versus mesothelioma (n = 42) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test. *p < 0.05

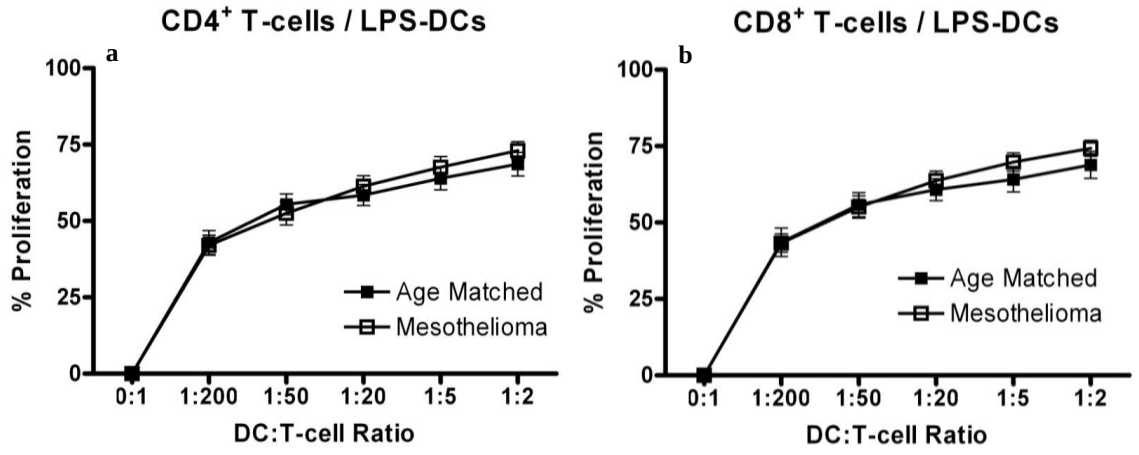

Figure 4.9: LPS-matured DCs mesothelioma maintain their ability to induce T cell proliferation

LPS activated MoDCs were co-cultured with allogeniec CFSE labelled lymphocytes for 7 days. Cells were collected, stained for CD4 and CD8 expression and analysed by flow cytometry. Lymphocytes were gated as either $CD4^+$ or $CD8^+$. The percentage of proliferating cells of the total gated population was determined. Pooled percentage proliferation was plotted against DC:T-cell ratio for age matched (n = 12) versus mesothelioma patients (n = 21) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test.

3.10 a-c). LPS-stimulated MoDCs from patients with mesothelioma secreted a complex pattern of cytokines; i.e. a trend towards increased levels of the pro-inflammatory cytokines TNF (Figure 4.10e: p = 0.11) and IL-12p70 (Figure 4.10h: p = 0.19). No differences were observed for the secretion of the pro-inflammatory cytokine, IFNγ (Figure 4.10i: p = 0.48), the anti-inflammatory cytokine IL-10 (Figure 4.10f: p = 0.42) and the pro-angiogenic soluble factor, VEGF (Figure 4.10g: p = 0.85) relative to the controls. Unfortunately this study was unerpowerd to detect a significant difference in TNF and IL-12. Futher studies with a larger population could identify whether the increased pro-inflammatory cytokines contribute to the improved LPS-driven DC maturation seen in mesothelioma patients.

4.2.10 IFNγ-stimulated mesothelioma MoDCs express lower levels of CD83

Immature MoDCs derived from patients with mesothelioma and healthy controls were incubated for 48 hours with IFNγ (Figure 4.11a). No differences were seen in either the percentage of $CD14^-$ cells expressing $CD11c^+$ (Figure 4.11b: p = 0.32) or CD11c surface expression levels (Figure 4.11c: p = 0.88). Similarly, no differences were seen in the percentage of $CD14^-$ cells expressing CD40 (Figure 4.11d; p = 0.96) or CD86 (Figure 4.11f: p = 0.20) or the surface expression levels of CD40 (Figure 4.11e: p = 0.34) and CD86 (Figure 4.11g: p = 0.45). However, there was a significant decrease in the percentage of cells expressing CD83 (Figures 4.11h: p = 0.021) with expression levels of CD83 also trending downwards (Figure 4.11i: p = 0.23). These data imply a partial maturation block in mesothelioma DCs in response to IFNγ.

IFNγ-activated MoDCs were further gated into $CD1a^+CD11c^+$ DCs. No differences were observed for either the percentage of $CD11c^+$ DCs expressing CD1a (Figure 4.12a: p = 0.70) or CD1a surface expression levels (Figure 4.12b: p = 0.54) between mesothelioma patients and healthy controls. No differences were observed for the percentage of $CD1a^+CD11c^+$ DCs expressing HLA-DR (Figure 4.12c: p = 0.98) or CD80 (Figure 4.12e: p = 0.19). However, a slight decreasing trend was observed for the surface expression levels of both HLA-DR (Figure 4.12d: p = 0.097) and CD80 (Figure 4.12f: p = 0.066).

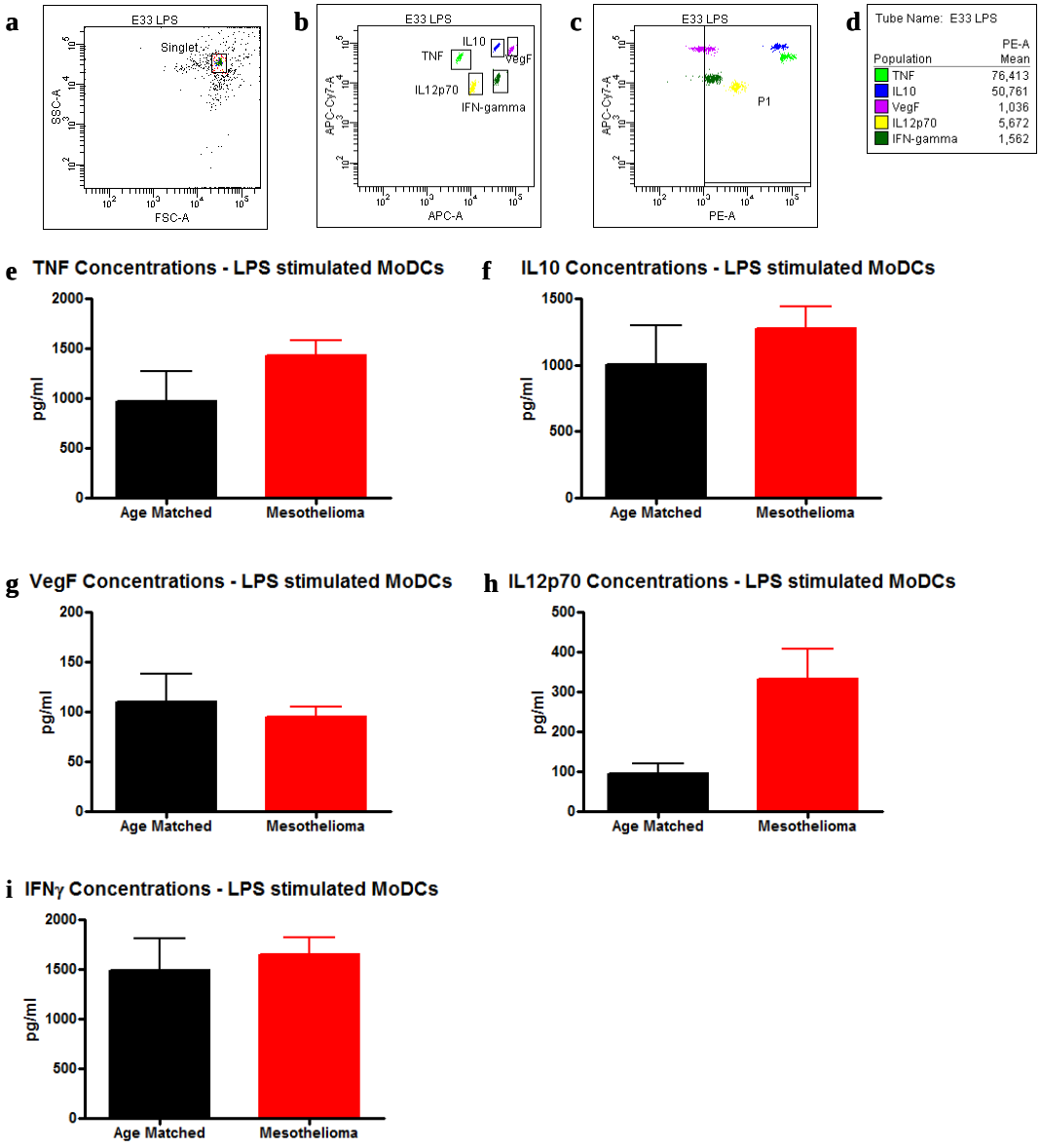

Figure 4.10: LPS-activated MoDCs from mesothelioma patients show an increased trend to secrete TNF, IL-12p70 and IL-10

Culture media from LPS-stimulated MoDCs from mesothelioma patients and healthy age matched controls were analysed by cytometric bead array for the production of cytokines. Representative plot (a) showing gating of pooled beads by size and granularity. Beads were further gated (b) to identify each cytokine tested. The concentration of each cytokine present was determined by measuring MFI (c,d) and correlating it to a standard curve. Pooled data for the concentration of TNF (e), IL-10 (f), VEGF (g), IL-12p70 (h) and IFNγ (i) secreted by MoDCs from mesothelioma patients (n = 45) and age matched controls (n = 14). Pooled data is shown as mean ± SEM.

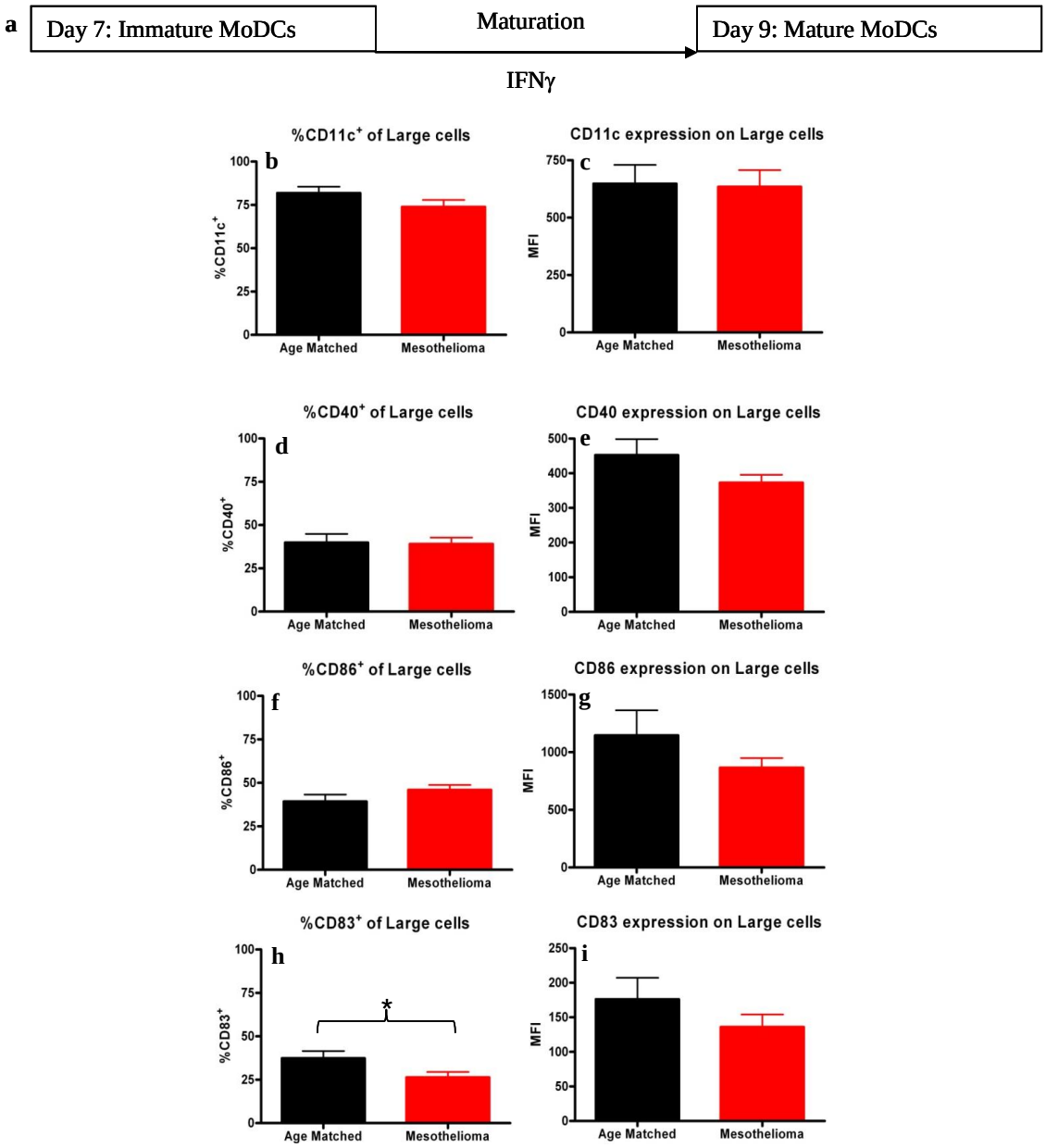

Figure 4.11: IFNγ-matured MoDCs from mesothelioma patients express lower levels of CD83 compared with age-matched controls

Immature MoDCs generated from mesothelioma patients and age-matched volunteers were stimulated with IFNγ (a) and cell surface molecules analysed by flow cytometry. Pooled data of the percentages of cells positive for CD11c (b), CD40 (d), CD86 (f) and CD83 (h). Surface expression levels were measured and shown as MFIs of CD11c (c), CD40 (e), CD86 (g) and CD83 (i) in mesothelioma patients (n = 46) versus age-matched (n = 26) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test. *p < 0.05

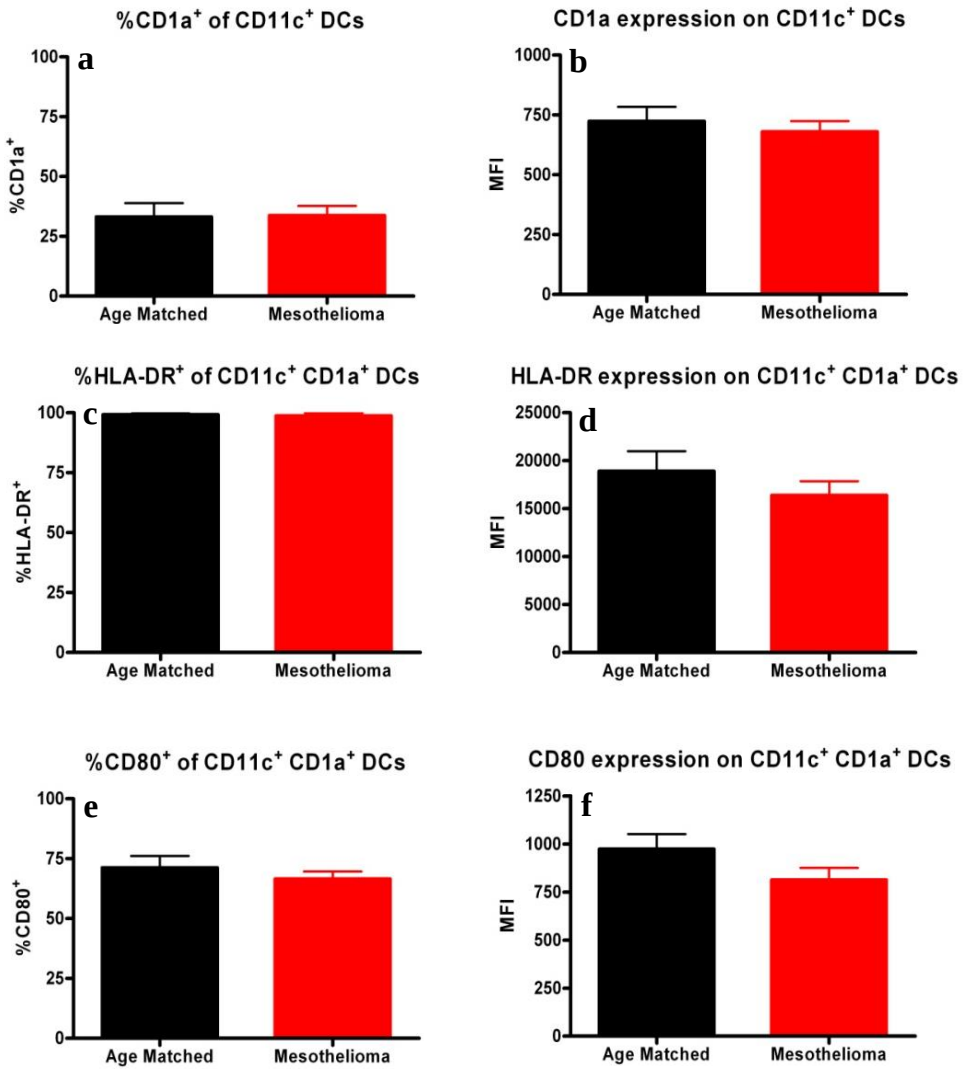

Figure 4.12: MoDCs from mesothelioma patients have a decreased trend in expression of CD80 and HLA-DR following exposure to IFNγ

Immature MoDCs generated from mesothelioma patients and age matched volunteers were stimulated with IFNγ and cell surface molecules analysed by flow cytometry. Large cells were identified and $CD11c^+$ cells gated. $CD11c^+$ DCs were analysed for the percentage of cells that were $CD1a^+$ (a) and for CD1a surface expression levels (MFI; b). Pooled data for the percentage of $CD11c^+CD1a^+$ DCs cells positive for HLA-DR (c) and CD80 (e) and for cell surface expression levels (MFIs) of HLA-DR (d) and CD80 (f) is from mesothelioma patients (n = 46) versus age matched (n = 26) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test.

4.2.11 IFNγ-matured MoDCs derived from people with mesothelioma do not fully lose their capacity to process antigen

Data in chapter 3 showed that MoDCs from elderly volunteers undergo maturational paralysis following stimulation with IFNγ. Unlike young healthy donors, greater than 75% of the MoDCs from elderly controls (56 to 84 years of age) retained their ability to process antigen and the relative antigen processing capacity was only reduced by approximately 50%. Interestingly, whilst the capacity to process antigen (measured as MFI) by MoDCs from patients with mesothelioma was significantly less than for age matched controls, their ability to process antigen was similar to the age-matched controls (Figure 4.13b) and the level of their antigen processing capacity was reduced also by approximately 50% (Figure 4.13c). These data imply that mesothelioma-derived MoDCs have the same impairment to IFNγ maturation as their healthy age-matched counterparts and that the apparent improvement (Figure 4.13a) reflects the initial impairment at the immature stage.

4.2.12 Host mesothelioma does not alter the capacity of IFNγ-matured DCs to induce T cell proliferation

IFNγ-matured MoDCs from 21 patients with mesothelioma and 12 healthy volunteers were examined for their ability to induce T cells from the universal allogeneic healthy donor to proliferate as previously described. No differences were noted between IFNγ-matured MoDCs from patients versus age-matched controls (Figures 4.13d and 4.13e).

4.2.13 IFNγ-activated MoDCs from people with mesothelioma secrete equal levels of cytokines in comparison to healthy age-matched controls

Conditioned media from IFNγ-activated MoDCs was analysed for cytokines by cytometric bead array. Both healthy-derived and patient-derived MoDCs secreted only minimal amounts of cytokines following IFNγ activation with IL-10, IL-12p70 and VEGF concentration lower than the detectable limit of the assay (Data not shown). No

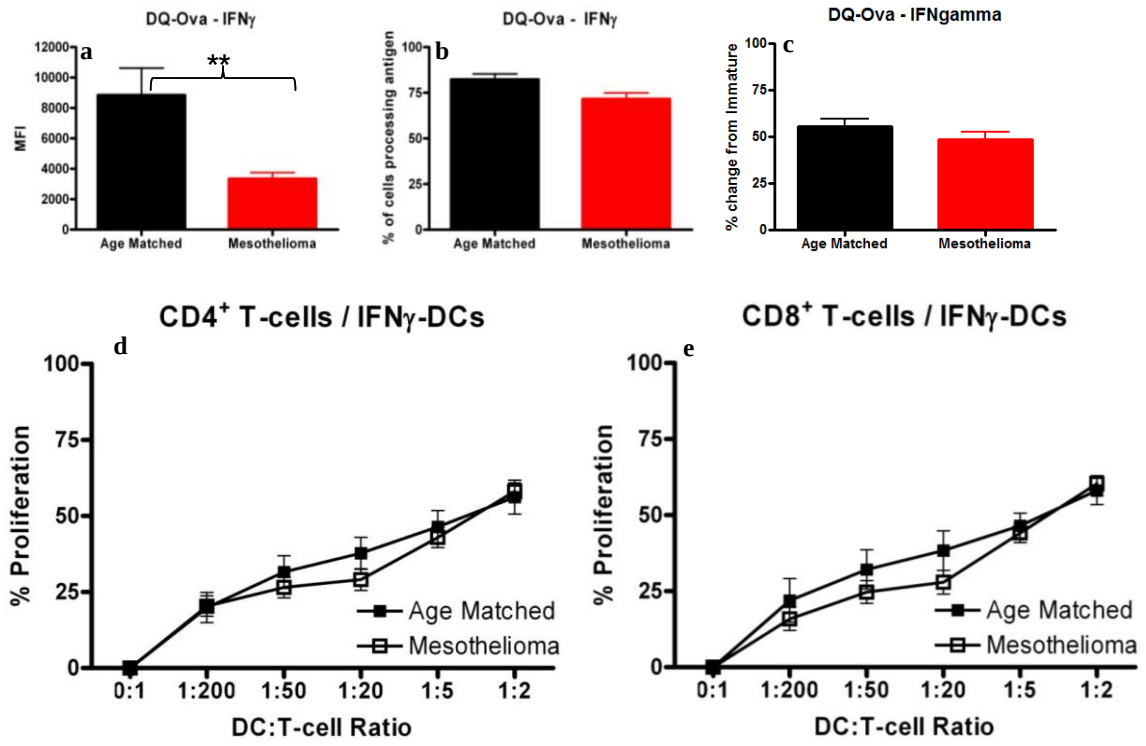

Figure 4.13: IFNγ matured-MoDCs from mesothelioma patients do not fully lose their capacity to process antigen but are still fully capable of inducing $CD4^+$ and $CD8^+$ T cell proliferation

MoDCs from mesothelioma patients and healthy age-matched volunteers previously stimulated with IFNγ, were incubated for 1 hour with FITC-DQ-Ovalbumin (DQ-OVA) as per Figure 4. The capacity to process antigen was measured by flow cytometric analysis. Pooled data of MFIs indicating relative antigen processing capacity (a), the percentage of DCs still processing antigen (b) and the percentage change from immature for IFNγ-treated age-matched (n = 29) versus mesothelioma (n = 42) MoDCs is shown as mean ± SEM. IFNγ-activated MoDCs were co-cultured with allogeniec CFSE- labelled lymphocytes for 7 days. Cells were collected, stained for CD4 (d) and CD8 (e) expression and analysed by flow cytometry. The percentage of proliferating cells of the total gated population was determined. Pooled percentage proliferation was plotted against DC:T-cell ratio for age-matched (n = 12) versus mesothelioma patients (n = 21) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test. $^{**}p < 0.01$, $^{*}p < 0.05$

significant difference was observed for TNF (Figure 4.14a: $p = 0.16$) or IFNγ (Figure 4.14e: $p = 0.55$) secretion.

4.2.14 LPS+IFNγ stimulated MoDCs from patients do not upregulate CD86 compared with age match controls

MoDCs from 46 people with mesothelioma and 26 age-matched controls were co-exposed to LPS plus IFNγ for 48 hours (Figure 4.15a). No differences were observed in the percentage of $CD14^-$ cells that were $CD11c^+$ (Figure 4.15b: $p = 0.28$, $CD40^+$ (Figure 4.15d: $p = 0.49$), $CD86^+$ (Figure 4.15f: $p = 0.71$) or $CD83^+$ (Figure 4.15h: $p = 0.34$). Whilst stable expression levels were seen for CD83 (Figure 4.15i: $p = 0.12$), two markers, i.e. CD11c (Figure 4.15b: $p = 0.19$) and CD40 (Figure 4.15e: $p = 0.16$) demonstrated a downward trend on mesothelioma-derived MoDCs however, the differences did not reach statistical significance. In contrast, a significant decrease was observed for CD86 on mesothelioma MoDCs relative to age-matched controls (Figure 4.15g: $p = 0.044$). These data imply that mesothelioma MoDCs do not achieve full activation relative to their controls after LPS/IFNγ-activation.

LPS/IFNγ-matured MoDCs were further gated as $CD11c^+$ DCs and examined for CD1a expression. No differences were seen in the percentage of $CD11c^+$ cells which were $CD1a^+$ (Figure 4.16a: $p = 0.89$), or in the percentage of $CD11c^+CD1a^+$ cells that were HLA-DR^+ (Figure 4.16c: $p = 0.73$) or $CD80^+$ (Figure 4.16e: $p = 0.87$). However, a trend was observed for the surface expression levels of CD1a (Figure 4.16b: $p = 0.73$) on $CD11c^+$ cells, HLA-DR (Figure 4.16d: $p = 0.14$), and CD80 (Figure 4.16f: $p = 0.19$) surface expression levels to be decreased on MoDCs from people with mesothelioma.

4.2.15 LPS/IFNγ-matured-MoDCs from patients exhibit a greater loss of antigen processing than healthy MoDCs and present antigen to T cells

LPS/IFNγ-matured MoDCs from 44 people with mesothelioma and 25 healthy age-matched controls were analysed for their ability to process DQ-OVA. Greater than 75% of mesothelioma-derived LPS/IFNγ-matured MoDCs lost their ability to process antigen (Figure 4.17a; $p = 0.067$), this was matched by lower expression levels of degraded

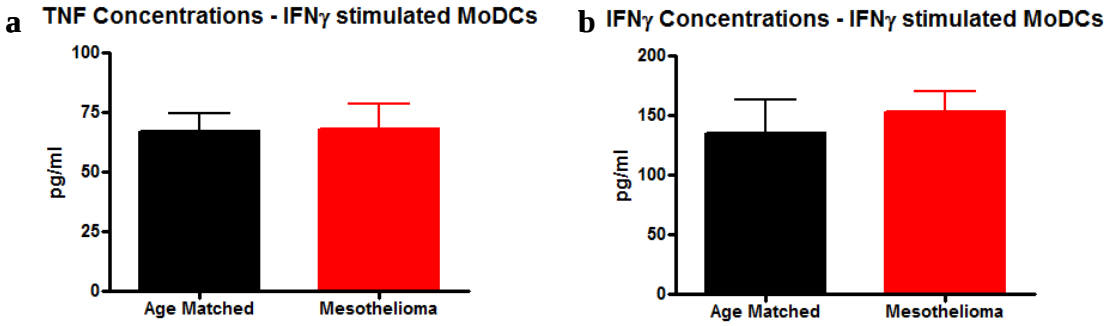

Figure 4.14: IFNγ-activated MoDCs from mesothelioma patients secrete equal levels of cytokines in comparison to healthy age matched controls

Culture media from IFNγ-stimulated MoDCs from mesothelioma patients and healthy age matched controls were analysed by cytometric bead array for the production of cytokines. Cytokine concentration was determined by measuring the MFI of the corresponding beads as per Figure 10. Pooled data for the concentration of TNF (a) and IFNγ (b) secreted by MoDCs from mesothelioma patients (n = 45) and age matched controls (n = 14). Pooled data is shown as mean ± SEM.

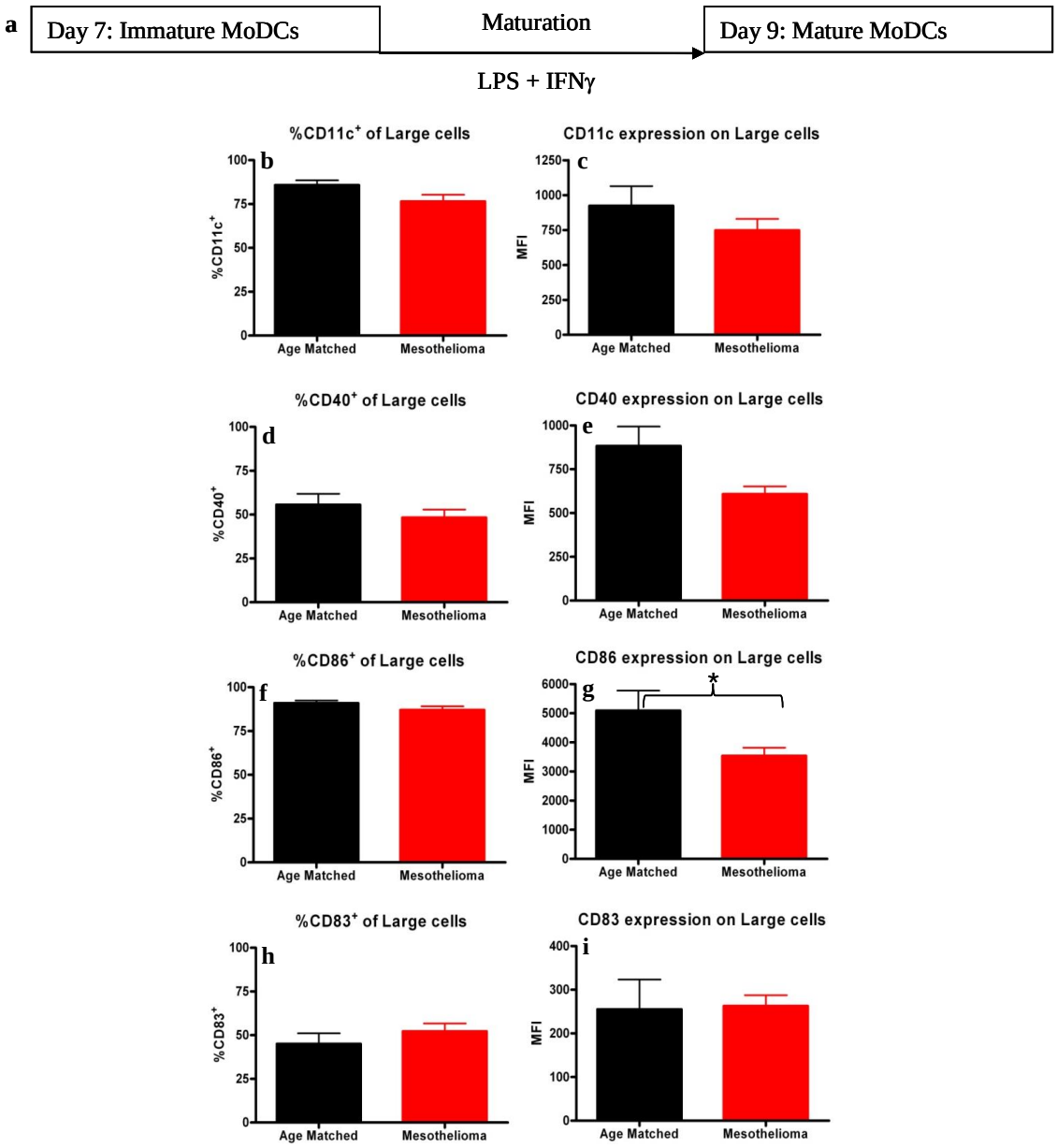

Figure 4.15: CD86 expression is decreased in LPS/IFNγ-stimulated MoDCs in mesothelioma patients compared with age matched controls

Immature MoDCs generated from mesothelioma patients and age-matched volunteers were stimulated with LPS + IFNγ (a) and cell surface molecules analysed by flow cytometry. Pooled data of the percentages of cells positive for CD11c (b), CD40 (d), CD86 (f) and CD83 (h). Surface expression levels were measured as MFIs of CD11c (c), CD40 (e), CD86 (g) and CD83 (i) in mesothelioma patients (n = 46) versus age matched (n = 26) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test. *$p < 0.05$

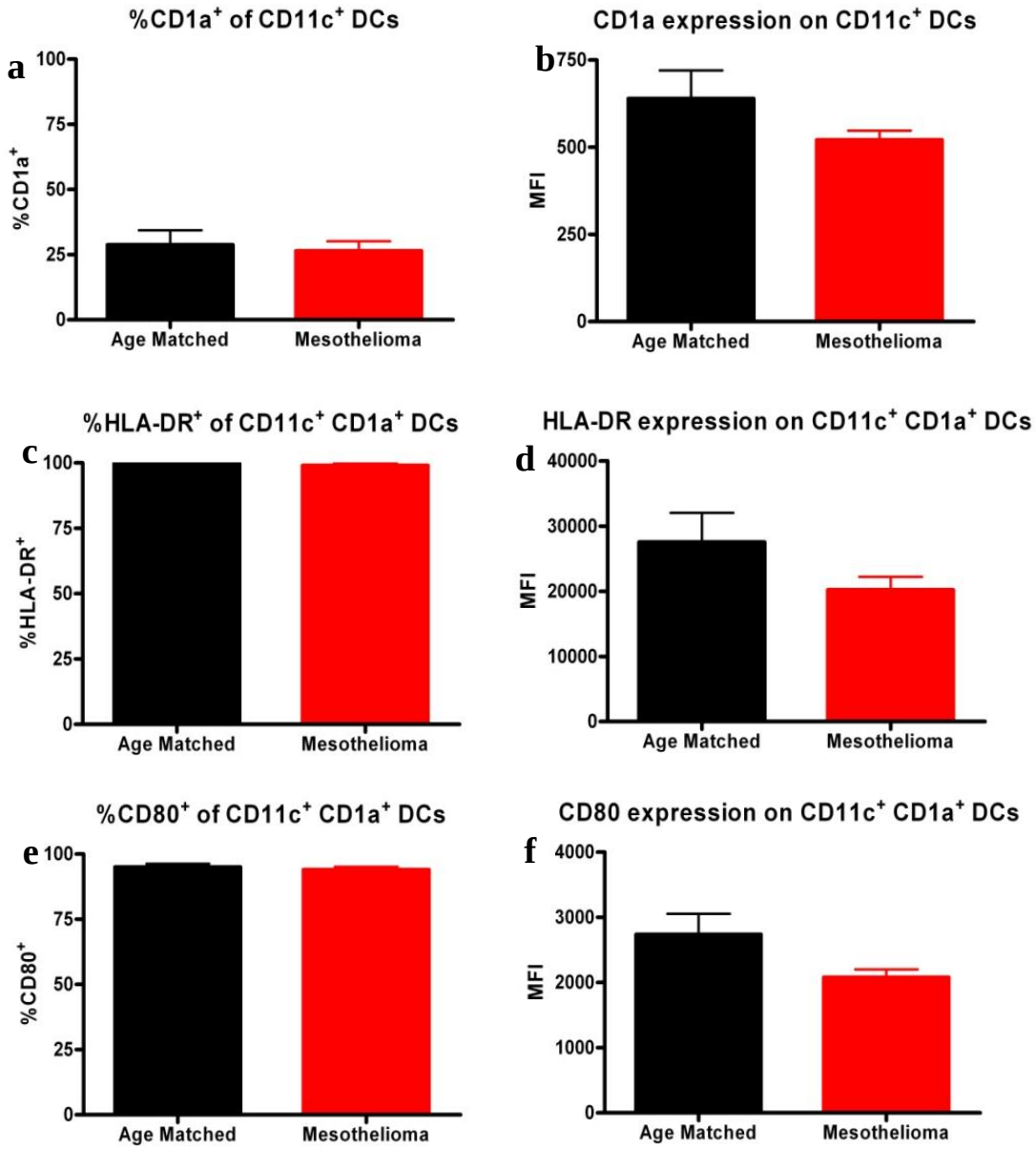

Figure 4.16: LPS/IFNγ-activated mesothelioma CD11c$^+$ MoDCs have lower expression levels of CD80 and HLA-DR

Immature MoDCs generated from mesothelioma patients and age-matched volunteers were stimulated with LPS and IFNγ and cell surface molecules analysed by flow cytometry. CD11c$^+$ DCs were analysed for the percentage of CD1a$^+$ cells (a) and CD1a expression levels (b). CD11c$^+$CD1a$^+$ DCs were analysed for expression of HLA-DR (c,d) and CD80 (e,f). Pooled data for the percentage of cells positive for CD1a (a), HLA-DR (c) and CD80 (e) and for cell surface expression levels (MFIs) of CD1a (b), HLA-DR (d) and CD80 (f) is from mesothelioma patients (n = 46) versus age-matched (n = 26) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

FITC-DQ-OVA (Figure 4.17b; p = 0.26). In contrast, only 50% of age-matched-derived MoDCs lost antigen processing ability. However, following stimulation with LPS and IFNγ a 90% decrease in antigen processing capacity (MFI) was observed irrespective of the MODCs originating from patients or healthy controls (Figure 4.17c; p = 0.26) indicating that the antigen processing ability of MoDCs from people with mesothelioma is already impaired at the immature stage of development. LPS/IFNγ-activated MoDCs from patients and age matched controls were also incubated with CFSE-labeled lymphocytes from the healthy universal donor. No differences were observed between MoDCs from patients or healthy volunteers in their capacity to induce $CD4^+$ (Figure 4.17d) and $CD8^+$ (Figure 4.17e) T cell proliferation.

4.2.16 LPS/IFNγ-activated MoDCs from patients secrete different levels of cytokines compared to age-matched controls

Conditioned media from LPS/IFNγ-activated MoDCs was analysed for cytokine content by cytometric bead array. No differences were observed between patients and controls for the secretion of VEGF (Figure 4.18c: p = 0.97) or IFNγ (Figure 4.18e: p = 0.72). One pro-inflammatory cytokine, TNF (Figure 4.18a: p = 0.51) demonstrated a marked reduction in concentration and decreased inter-person variability in people with mesothelioma, although wide variation in the aged matched controls meant that this did not reach statistical significant. A second pro-inflammatory cytokine, IL-12p70, (Figure 4.18d: p = 0.63) was observed to have a trend towards increased concentrations in people with mesothelioma, although there was substantial variability in patient levels precluding statistical significance. The anti-inflammatory cytokine IL-10 (Figure 4.18b: p = 0.12) also appeared to increase in mesothelioma patients.

4.2.17 The number of circulating mDC1s and certain responses of MoDCs to stimuli correlates with increased survival

To investigate whether the number of circulating DCs correlates with increased survival, patients were ranked according to the number of pDCs, mDC1s or mDC2s from which Kaplan-Meier plots were generated. Patients were dichotomised into those with a higher

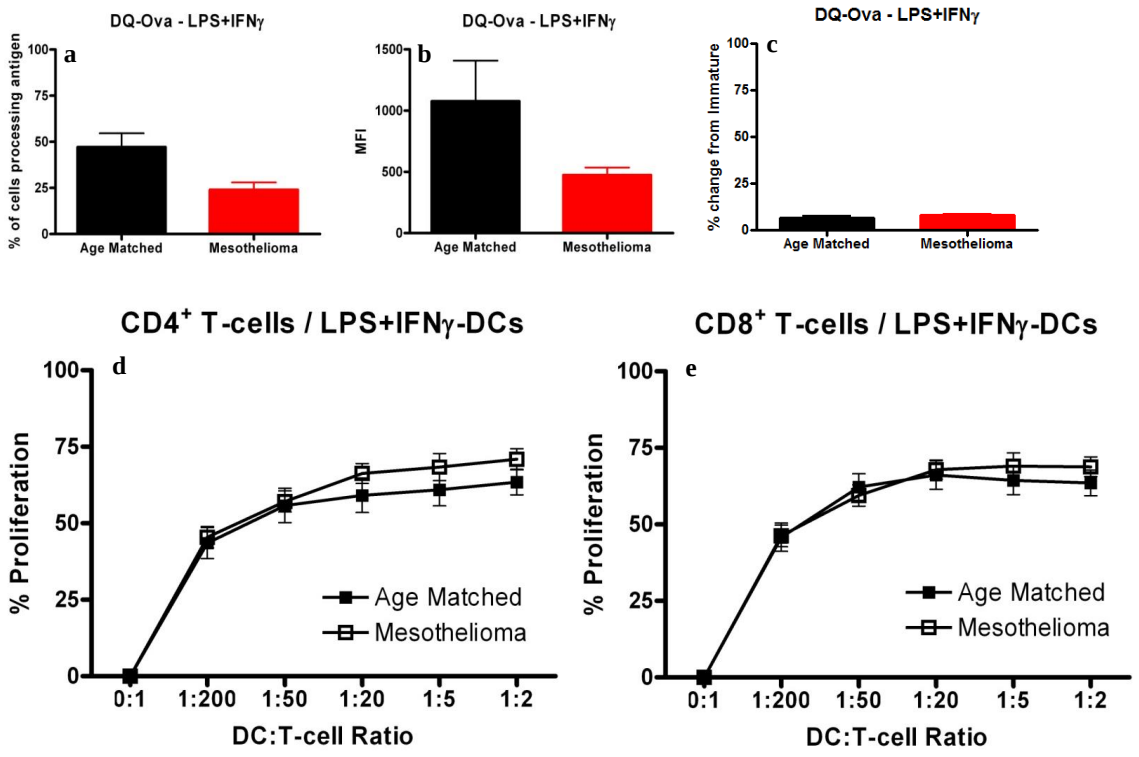

Figure 4.17: LPS/IFNγ-matured-MoDCs from mesothelioma patients exhibit a greater loss of antigen processing than healthy MoDCs and have no impairment in their ability present antigen to T cells

MoDCs from mesothelioma patients and healthy age-matched volunteers previously stimulated with LPS+IFNγ were incubated for 1 hour with FITC-DQ-Ovalbumin (DQ-OVA). The capacity to process antigen was measured by flow cytometric analysis. Pooled data of MFIs indicating relative antigen processing capacity (a), the percentage of DCs still processing antigen (b) and the percentage change from immature for LPS/IFNγ treated age-matched (n = 29) versus mesothelioma (n = 42) MoDCs. LPS/IFNγ-activated MoDCs were co-cultured with allogeniec CFSE-labelled lymphocytes for 7 days. Cells stained for CD4 (d) and CD8 (e) expression and analysed by flow cytometry. The percentage of proliferating cells of the total gated population was determined. Pooled percentage proliferation was plotted against DC:T-cell ratio for age-matched (n = 12) versus Mesothelioma patients (n = 21) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using the two-tailed Mann-Whitney test.

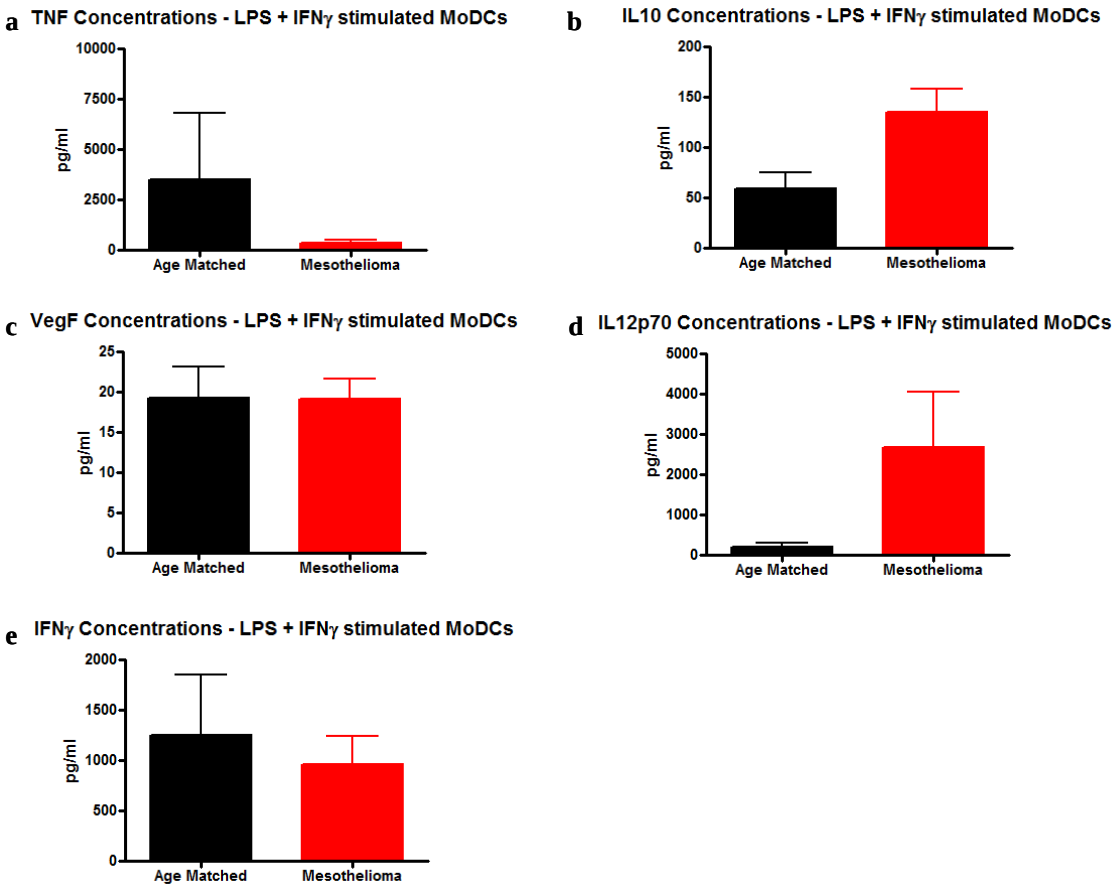

Figure 4.18: LPS/IFNγ-activated MoDCs from mesothelioma patients secrete different levels of cytokine compared to age matched controls

MoDCs from mesothelioma patients and healthy age match controls were stimulated with both LPS and IFNγ for 48 hours. Culture media was collected and analysed by cytometric bead array for the presence of cytokines. Cytokine concentration was determined by measuring the MFI of the corresponding beads as per Figure 10. Pooled data for the concentration of TNF (e), IL-10 (f), VEGF (g), IL-12p70 (h) and IFNγ (i) secreted by MoDCs from mesothelioma patients (n = 46) and age matched controls (n = 14) is shown as mean ± SEM.

than median number of DCs and those below the median. The number of pDCs and mDC2s in patients was observed to have no impact on survival (Data not shown). In contrast, patients with a higher than median number of circulating mDC1s were shown to have a significant increase in their survival time (Figure 4.19a).

Expression of surface markers, pre and post stimulation with LPS +/- IFNγ were also correlated against survival time. Expression of surface markers on iMoDCs, LPS matured MoDCs and LPS + IFNγ matured MoDCs showed no correlation with survival (Data not shown). From the IFNγ matured MoDCs, only patients with a higher than median expression of CD80 showed a significantly increased survival time (Figure 4.19b).

Similarly, antigen processing ability was analysed for any correlation with increased survival. Whilst antigen processing of iMoDCs, LPS-stimulated MoDCs and IFNγ-stimulated MoDCs had no correlation with increased survival (Data not shown), patients with a lower than median antigen processing ability post stimulation with LPS and IFNγ, indicating an improved response to stimulation, also had a significantly increased survival time (Figure 4.19c)

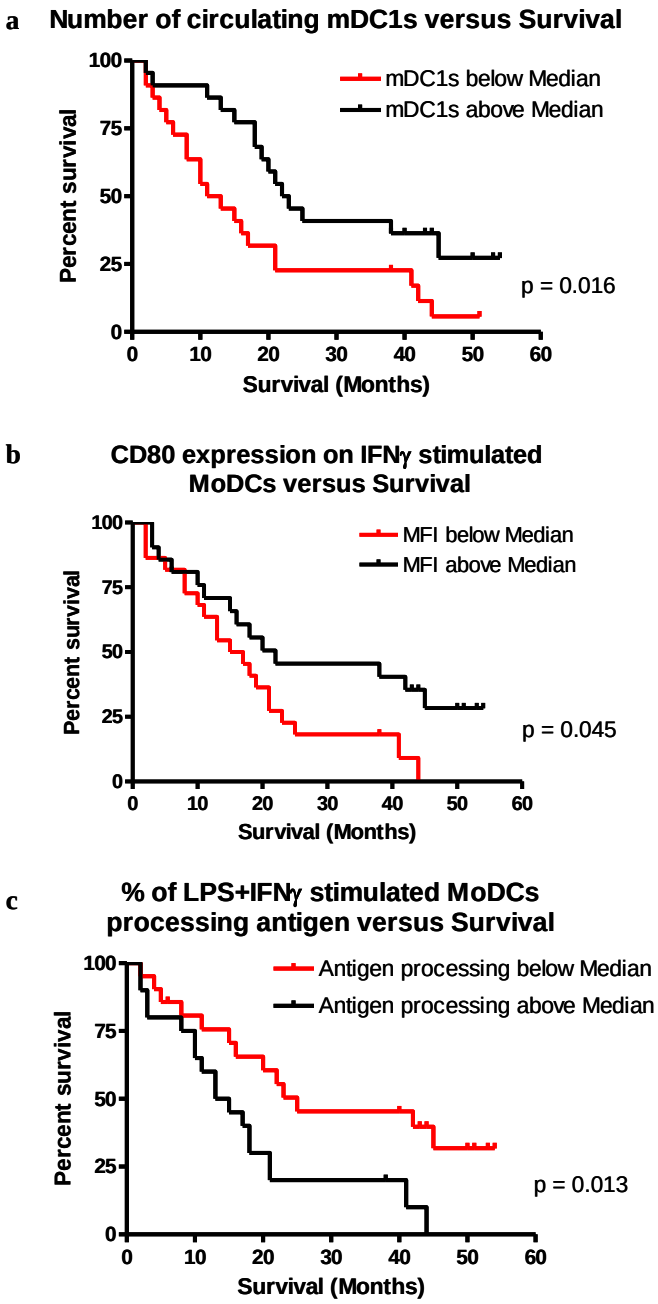

Figure 4.19: Data correlating with increased survival

Whole blood was stained and analysed for blood DC subpopulations as previously shown (Figure 4.1). The number of circulating MDC1s was plotted against survival (in months) from time of blood collection (a). Relative expression (MFI) of CD80 following stimulation with IFNγ was plotted against survival (b). The percentage of mesothelioma patient-derived LPS and IFNγ stimulated MoDCs able to process antigen was graphed against survival (c). p values were determined using Logrank Test.

4.3 Discussion

Dendritic cells are an important cell type for anti-tumour responses (Banchereau and Steinman, 1998, Byrne and Halliday, 2002). Their ability to take up and present antigens is critical in the initiation of antigen-specific T cell responses. Whilst there is no published data on the effect of mesothelioma on either the numbers of blood dendritic cells or any functional consequences, there have been several studies investigating the effect of a number of other cancers on blood DC subsets. Thus, the aim of this study was to investigate whether people with mesothelioma have defects in dendritic cell number or function.

Data from this study shows that mesothelioma affects pDCs similar to other cancers, including breast carcinoma, multiple myeloma, Kaposi sarcoma and pancreatic adenocarcinoma i.e. there is a significant decrease in pDC numbers, identified by expression of either CD123 (the low affinity alpha subunit of the IL-3 receptor) or CD303 (BDCA-2), in cancer patients (Della Bella et al., 2006, Harrison et al., 2008, Pinzon-Charry et al., 2007, Tjomsland et al., 2010). In contrast, studies examining squamous cell carcinomas have shown no change in the pDC population in patients irrespective of stage of disease (Hoffmann et al., 2002, Ma et al., 2009, Sakakura et al., 2006). It is possible that soluble or anatomical factors causing the down-regulation in pDCs numbers in mesothelioma and other cancers are not present in squamous cell carcinomas. However, cancer stage may play a role as Pinzon-Charry et al (2007) observed that decreases in pDC numbers only become significant in late stage patients. The visible nature of squamous cell carcinomas may mean that most diagnoses are made at an earlier stage of disease development than more silent cancers such as mesothelioma.

As mentioned in chapter 3, the use of a specific marker for myeloid dendritic cells was not consistent between studies. Studies investigating the effect of cancer on myeloid dendritic cells often used CD11c as their only surface marker which, instead of delineating the myeloid subsets, groups all myeloid DC subsets, including mDC1s,

mDC2s and MoDCs into a single population. Interestingly, these studies consistently observed a significant decrease in mDCs in people with a malignancy, irrespective of the cancer studied (Della Bella et al., 2006, Hoffmann et al., 2002, Ma et al., 2009, Pinzon-Charry et al., 2007, Sakakura et al., 2006, Tjomsland et al., 2010). This study observed a significant numerical decrease in circulating mDCs in people with mesothelioma, however, the use of more specific markers revealed significant decreases in both mDC1 and mDC2 populations. In contrast, the study by Harrison et al (2008), which used an identical selection of markers to differentiate the mDC1 and mDC2 populations, only observed a significant decrease in the mDC1 population; nonetheless a decreased trend for mDC2s was observed. In that study the sample size used was small (n = 7), and given that mDC2s make up less than 0.05% of leukocytes found in blood, it is likely that the small sample did not have the power to detect statistically significance even with a similar magnitude of difference to this study. Unlike previous studies in other cancers, which did not correlate number of circulating mDCs to survival, this study showed that patients with a higher than median number of circulating mDC1s had a significant increase in survival. These patients may represent a cohort of patients whose mDC1s are resistant to the tumours mechanisms of decreasing circulating DC numbers (i.e. increased apoptosis or decreased generation).

It is difficult to be certain how the alterations in numbers and function of blood DCs relates to DCs in tumours, as there are very few studies that have investigated the effect of cancer concurrently on both tumour DCs and circulating DCs in humans. Studies by Tabakiewicz et al. (2008) and Perrot et al. (2007) observed a decrease in mDCs in the peripheral blood, and a predominance for pDCs within the tumour microenvironment in lung cancer patients. Similarly Gigante et al. (2009), observed an increase in pDCs in the tumour microenvironment in renal carcinoma patients compared to healthy donor tissue. Both Perrot et al. and Gigante et al. characterized these pDCs as having an immature phenotype. Several other studies have linked the increase in pDCs in the tumour microenvironment with a poor prognosis, highlighting a tolerizing effect by the pDCs (Hartmann et al., 2003, Treilleux et al., 2004, Wei et al., 2005). Thus, the observed

results of a low number of circulating pDCs might only be in the periphery, and that this may be different to what would be observed in the tumour microenvironment.

As mentioned previously, due to the low numbers of circulating DCs functional changes were investigated using in vitro generated MoDCs. This study observed impairments in the ability of monocytes to differentiate into MoDCs in people with mesothelioma. Specifically, trends for reduced expression were observed in most surface markers (CD11c, CD80, CD83, CD86 and HLA-DR) following differentiation with GM-CSF and IL-4. A significant decrease was also observed in the expression of CD40, a key co-stimulatory molecule. Whilst not identical, similar deficiencies in surface marker expression have been observed in breast cancer, squamous cell carcinomas and hepatocellular carcinoma (Ma et al., 2009, Ninomiya et al., 1999, Pinzon-Charry et al., 2007, Sakakura et al., 2006). These data imply that mesothelioma, like other cancers, has the ability to regulate DC differentiation, possibly through the secretion of soluble factors, such as TGFβ (Gerwin et al., 1987) or RANTES (Hegmans et al., 2006).

A key function of immature dendritic cells is antigen uptake and processing which allows for the presentation of antigens to T cells following maturation. This study observed a deficiency in antigen processing in immature MoDCs from people with mesothelioma. Whilst the majority of mesothelioma-derived immature MoDCs demonstrated an ability to process antigen, the level of antigen processed was significantly reduced relative to the healthy age-matched controls. These data suggest that mesothelioma-derived MoDCs are either partially mature in the absence of a specific maturation signal or have a specific defect in their ability to process antigen. Pinzon-Charry et al (2007) observed that blood DCs from patients with predominantly early-stage breast cancer were significantly impaired in their antigen uptake ability. However, this contrasts with a study by Ninomiya et al (1999), which observed that MoDCs from patients with late-stage hepatocellular carcinoma had a particularly immature phenotype on account of their increased ability to take up antigen. The differences in these studies indicate that cancers may use different mechanisms to escape the immune system, or that the stage of cancer may impact of the observed effect.

This study showed that iMoDCs could induce allogeneic T cell proliferation, although not as effectively as mature MoDCs and that iMoDCs from mesothelioma patients were equally capable as healthy controls in inducing $CD4^+$ and $CD8^+$ T cell proliferation. Similarly, Gabrilovich et al. (1997) also observed that MoDCs derived from women with breast cancer responded as well as healthy controls in an allogeneic MLR. In contrast, both Pinzon-Charry et al. (2007) and Gabrilovich et al (1997) observed that blood DCs from patients with breast cancer had a decreased ability to induce T cell proliferation. It is possible that in vitro differentiation generates DCs with normal T cell proliferative capabilities, whilst in vivo differentiation under the induced pressure of tumour factors generates a DC with impaired T cell proliferative capabilities. Further studies would be required to address this.

This study also examined immature MoDC responses to LPS and/or IFNγ. Following exposure to LPS alone, a decreased trend in the expression levels of all surface markers examined (i.e. CD1a, CD11c, CD40, CD80, CD83, CD86 and HLA-DR) was observed in MoDCs from mesothelioma patients implying an inability to fully mature. Similarly, when LPS was replaced with IFNγ decreased trends were observed in the expression levels of CD40, CD86, CD83, HLA-DR and CD80, with a significant decrease observed in the percentage of cells expressing CD83. As CD83 is a marker of maturation it is possible that MoDCs from mesothelioma patients do not complete the maturation process following exposure to IFNγ. The combination of LPS and IFNγ was not synergistic and produced marginally improved maturation relative to LPS or IFNγ alone, with only CD83 expression in MoDCs from patients matching that of healthy controls. However, whilst the CD83 response improved, CD86 expression for patient-derived MoDCs was significantly less than that of healthy controls. The data suggests that immature MoDCs generated from people with mesothelioma cannot fully respond to maturation signals and only achieve partial maturation. Interestingly, mesothelioma patients who showed and increased expression of CD80 following IFNγ stimulation had an increased survival time. The role of CD80 as a costimulatory factor may be aiding these patients survival by presenting tumour antigens to naïve T cells. These data contrast to Pinzon-Charry (2007) who observed that blood DCs from early-stage breast

cancer patients readily upregulated both CD86 and HLA-DR similar to healthy controls in response to a cytokine cocktail (IL-1β, IL-6, TNF-α and PGE_2) or poly I:C. It is important to note that the stimulated MoDCs originated from women with early-stage (pre-operation) breast cancer and Pinzon-Charry had previously only observed phenotypical changes in late-stage patients. In a study by Onishi et al. (2002) people with late stage cancers demonstrated decreased expression of CD80 following stimulation with conditioned media. These data show that the disease stage impacts on DC dysfunction. However, it should be noted that the stage of disease for mesothelioma is regarded as less important than for many other cancers. This is due to the fact that even early diagnosis of patients is not considered curable.

During maturation DCs lose their ability to process antigen and, as mentioned above, iMoDCs from people with mesothelioma may either be already maturing or have an underlying defect in antigen processing as they demonstrated a decreased ability to process antigen relative to the healthy controls in the absence of a maturation stimulus. Following activation with LPS, MoDCs from patients appeared to readily lose their antigen processing capacity and the percentage of MoDCs still able to process antigen was significantly less than that of age matched controls, but the relative change from the immature state was approximately the same. These data indicate that the observed lowered ability to process antigen in iMoDCs is due to a defect in immature DCs rather than premature maturation, as they responded to LPS to a similar degree to the age matched controls. Yet a form of maturation paralysis occurs as these cells were unable to upregulate key surface molecules. Following stimulation of MoDCs with IFNγ the relative decrease in antigen processing was similar to the healthy age matched controls indicating that the age-related paralysis in response to IFNγ is still present in mesothelioma patients. Similar to LPS alone, the combination of LPS/IFNγ yielded similar results to the healthy age matched controls. Although patients whose antigen processing capacity following stimulation with LPS and IFNγ was lower than median showed a significant increase in survival. How this aids survival, as iMoDCs appear dysfunctional in antigen processing, warrents further investigation, but may indicate that these patients have a small portion of DCs that resist impairment. The data indicates that

in the absence of stimuli, the antigen processing capacity of MoDCs from people with mesothelioma is already impaired. Taken together these data suggest that MoDCs from people with mesothelioma cannot fully mature partly due to their inability to fully up-regulate surface co-stimulatory molecules and maturation molecules, as well as due to a pre-existing defect in antigen processing.

Although defects were seen in up-regulation of co-stimulatory molecules, MoDCs from people with mesothelioma demonstrated an equivalent capacity to present antigen at sufficient levels to induce allogeneic $CD4^+$ and $CD8^+$ T cell proliferation. This response was irrespective of the stimuli used. In contrast, Pinzon-Charry et al. (2007) and Ma et al. (2009) observed a significant decrease in the ability of blood DCs and MoDCs from cancer patients stimulated with either a cytokine cocktail, poly I:C or TNFα to induce T proliferation. There are several differences between this study and the previous studies which could account for the observed differences. Firstly, Pinzon-Charry et al. used blood DCs rather than MoDCs and whilst both populations have been shown to be impaired by the presence of cancer, no side-by-side studies have been performed. Secondly, Ma et al. performed an autologous MLR rather than an allogeneic MLR. Whilst the use of autologous T cells allows for antigen specific proliferation to be determined, it introduces a new variable as it does not take into consideration that the disease has already impaired T cell function. Lastly, the present study has shown that age-related factors can impair DC responses to specific stimuli (i.e. elderly DCs respond poorly to IFNγ); the above mentioned studies used different stimuli to mature their DCs. In a review by Castiello et al. (2011), it has been shown that different stimuli act through specific cell signaling pathways and that the combination of stimuli can generate specific T cell subsets. Further studies are required to determine the specific subsets of T cells generated by MoDCs from mesothelioma patients.

MoDCs respond to activation by secreting cytokines (Shortman and Liu, 2002). LPS-activated mesothelioma patient-derived MoDCs secreted increased levels of the pro-inflammatory cytokines TNF and IL-12p70 as well as the anti-inflammatory cytokine IL-10 relative to healthy LPS-activated MoDCs. The increased production of both TNF

and IL-12p70 indicates the potential for strong cell mediated immune responses. Yet, the increased secretion of IL-10 indicates a suppressive effect. McBride et al. (2002) observed that DCs matured by LPS in the presence of IL-10 had reduced expression of co-stimulatory molecules. This could explain the down-regulation of co-stimulatory molecules seen on MoDCs from people with mesothelioma in this study. The presence of two populations of MoDCs producing either anti-inflammatory cytokines or pro-inflammatory cytokines in people with newly diagnosed mesothelioma may indicate a time point in disease progression where the immune escape is occuring. Whilst the DCs expressing IL-12 have the potential to activate T cells, other DCs (expressing IL-10) express low levels of costimulatory molecules to possibly inducing regulatory T cells. Della Bella et al (2006), observed a decrease in IL-12 with an increase in IL-10 in patients with Kaposi sarcoma indicating a strong pro-tumourigenic environment. As the underlying cause of Kaposi sarcoma is human herpesvirus-8, the presence of sarcomas could already indicate a failure in the immune system's ability to keep the virus in check.

Responses following activation with the combination of LPS and IFNγ were complex, unexpected and rather than being synergistic may have been antagonistic. For example, LPS/IFNγ-activated mesothelioma patient-derived MoDCs no longer secreted large concentrations of TNF, as seen when activated with LPS alone. The TNF concentrations secreted were also considerably less than healthy age-matched MoDCs. In contrast, whilst IL-10 secretion was higher than age matched-MoDCs, it was 10-fold less than that observed with LPS alone. Interestingly, IL-12p70 secretion dramatically increased and the absolute IL-12p70 concentration was a log-fold higher than the IL-10 concentration which may override any suppressive effects. Thus, the combination of LPS and IFNγ may be a sufficient stimulus to induce a strong anti-tumour response. These data imply that DCs from people with mesothelioma could be rescued if given the correct stimuli.

This study has shown that people with mesothelioma have decreased numbers of blood pDCs, mDC1s and mDC2s indicating a possible impairment in DC differentiation from

bone marrow precursor DCs or tumour-induced cell death. *In vitro* generation of DCs from blood monocyte precursors resulted in MoDCs with decreased co-stimulatory molecules in comparison to age matched controls further implying problems in DC differentiation that might be slightly improved likely due to the removal of suppressive factors. Even with the removal of suppressive factors, immature MoDCs from people with mesothelioma were unable to process antigen to the same levels as their healthy counterparts, implying tumour-induced dysfunction and premature activation. Although stimulation with LPS and/or IFNγ induced the maturation process it was incomplete as the decrease in antigen processing as expected of a maturing DC was only partial. Much of this maturational paralysis could be attributed to age-related issues as the responses were no better or worse than that seen in age matched controls which already have a defective response to IFNγ. Further evidence of incomplete maturation included reduced up-regulation of co-stimulatory molecules. Despite only achieving partial maturation, the MoDCs from people with mesothelioma demonstrated no defects in their ability to induce allogeneic T cell proliferation.

These data imply that people with mesothelioma have significant defects in their DC subsets, with reduced numbers of circulating blood DCs and specific impairments in their differentiation, antigen processing ability and upregulation of co-stimulatory molecules. Whilst antigen presentation is not impaired, a reduced ability to process antigen suggests that only limited antigen-specific responses would be generated and that the T cell response may be tolerised. Interestingly, there appears to be several factors that indicate an increase in survival. These include an increased number of circulating mDC1s, a higher up-regulation of CD80 following IFNγ stimulation and a stronger down-regulation of antigen processing following stimulation with LPS and IFNγ. The remainder of this thesis investigates whether soluble factors collected from mesothelioma cell lines induce similar defects in healthy MoDCs and whether impaired DC function can be restored by activation using CD40L.

5 CAN IMMUNOSENESCENT DCS BE RESCUED BY CD40L

5.1 Introduction

The studies presented in chapter 3 showed a significant decrease in circulating pDCs numbers with mDCs numbers remaining at normal numbers in elderly volunteers. Whilst monocytes from elderly individuals readily differentiated into MoDCs they responded poorly to stimulation with LPS or IFNγ. In particular, after IFNγ stimulation elderly-derived DCs maintained their antigen processing capacity indicating partial maturation paralysis.

CD40 is a transmembrane glycoprotein belonging to the Tumour Necrosis Factor Receptor superfamily (Banchereau et al., 1994). Whilst first identified on B cells, it has since been shown to be expressed on many different cell types including non-immune cells (epithelial and endothelial cells), immune cells (activated T cells, macrophages and DCs) and tumour cells (Grewal and Flavell, 1996, Stout and Suttles, 1996, Van Kooten and Banchereau, 1996). Similarly, CD40 ligand (CD154, CD40L) is also expressed on a wide variety of cells, including DCs, monocytes and activated T cells (Filion et al., 2003, Grewal and Flavell, 1998, Klaus et al., 1997).

During antigen presentation, the interaction between CD40 on DCs and CD40L on T cells leads to increased DC survival, up-regulation of co-stimulatory and MHC molecules, and an increase in cytokine production. This in turn drives the induction of highly activated cytotoxic T lymphocytes (CTLs). In the absence of endogenous T cell "help", exogenous CD40L or agonist anti-CD40 monoclonal antibody license DCs such that they induce a potent CTL response against tumours (Bennett et al., 1998, Fransen et al., 2011, Schoenberger et al., 1998, Toes et al., 1998). Thus, activating DCs through surface CD40 has been extensively examined as a potential anti-cancer immunotherapy, with promising results shown mainly in animal studies (Jackaman and Nelson, 2012, Khong et al., 2012) that used young adult mice. It is not yet clear if this approach would be as effective in older cancer patients.

The studies in this chapter aimed to determine whether CD40 activation could restore the age-related MoDC dysfunction shown in chapter 3.

5.2 Results

5.2.1 CD40-stimulation increases CD1a, CD40 and CD86 expression in healthy elderly MoDC

The first series of experiments investigated whether CD40 activation overcomes the age-related defects seen in response to stimuli shown in chapter 3. For these experiments healthy volunteers were dichotomised into two age groups as per chapter 3: i.e. young volunteers were aged 20 - 45 years old, whilst 'elderly' volunteers were 60 - 85 years old (see Table 3.1). Immature MoDCs from healthy volunteers were stimulated with CD40L for 48 hours (Figure 5.1a). Following CD40 activation and staining, cells were first gated by size (Figure 5.1b), and confirmed to be $CD14^-$ before investigating the expression of CD11c (Figure 5.1c), CD40 (Figure 5.1f), CD83 (Figure 5.1m) and CD86 (Figure 5.1j). $CD11c^+$ DCs were further investigated for the expression of CD1a (Figure 5.2c), CD80 (Figure 5.2i) and HLA-DR (Figure 5.2f).

Whilst no age related differences were observed for the percentage of $CD14^-$ cells expressing CD11c (Figure 5.1d; $p = 0.38$) and CD83 (Figure 5.1m; $p = 0.60$), a significant increase was observed for the percentage of large cells expressing CD40 (Figure 5.1g; $p = 0.015$) and CD86 (Figure 5.1j; $p = 0.0011$) in elderly-derived MoDCs relative to young-derived MoDCs. No age related differences were observed for the relative expression (MFI) of CD11c (Figure 5.1e; $p = 0.86$), CD40 (Figure 5.1h; $p = 0.46$) and CD83 (Figure 5.1n; $p = 0.65$). In contrast, a significant increase in expression of CD86 (Figure 5.1k; $p = 0.0035$) was observed in elderly-derived MoDCs.

Following gating on $CD11c^+$ cells, no age-related difference was seen in surface expression levels of CD11c (Figure 5.2e; $p = 0.65$), although the percentage of elderly-derived $CD11c^+$ MoDCs expressing CD1a was significantly increased relative to their younger counterparts (Figure 5.2d; $p = 0.045$). Investigation of the $CD11c^+CD1a^+$ DC subset did not reveal age-related differences in the percentage of cells expressing CD80 (Figure 5.2j; $p = 0.38$) or HLA-DR (Figure 5.2g; $p = 0.32$). Likewise, no age-related

differences were observed for surface expression levels of CD80 (Figure 5.2k; $p = 0.15$) or HLA-DR (Figure 5.2h; $p = 0.20$).

Comparisons between post-CD40L stimulation and unstimulated MoDCs revealed significant increases for the percentage of elderly-derived MoDCs expressing CD40 (Figure 5.1g; $p = 0.0017$) and CD86 (Figure 5.1j; $p < 0.0001$). Likewise, a significant increase was observed for surface expression levels for CD40 (Figure 5.1h; $p = 0.027$) and CD86 (Figure 5.1k; $p = 0.0002$) on elderly-derived MoDCs. In contrast, CD40 and CD86 expression levels significantly decreased (Figure 5.1k; $p = 0.039$) on young-derived MoDCs following CD40L stimulation.

5.2.2 Stimulation of elderly-derived MoDCs with CD40L overcomes the antigen processing paralysis observed following LPS +/- IFNγ stimulation

The results from chapter 3 showed that age had no impact on the antigen processing ability of immature MoDCs. However, following LPS +/- IFNγ stimulation, unlike young derived MoDCs, elderly-derived MoDCs maintained their antigen processing ability. These data imply that elderly-derived MoDCs did not fully mature in a similar manner to the young-derived DCs. To investigate whether CD40L activation induced full DC maturation, the DQ-Ova assay was used to compare the antigen processing capacity of CD40L stimulated MoDCs derived from 12 young and elderly healthy individuals. MoDCs were first gated for by size (Figure 5.3a) before measuring the degradation of FITC-labelled DQ-Ovalbumin (Figure 5.3b). Following CD40L stimulation, the percentage of young-derived MoDCs (Figure 5.3c; $p < 0.0001$) and elderly-derived MoDCs (Figure 5.3c; $p < 0.0001$) still processing antigen significantly decreased. Likewise the amount of antigen processed significantly decreased for both age groups (Figure 5.3d; Young: $p = 0.0020$; Elderly: $p = 0.0012$). Interestingly, after CD40 activation, significantly fewer elderly-derived MoDCs retained their capacity to process antigen (Figure 5.3c; $p = 0.019$). Nonetheless, the MoDCs that could process antigen did so at similar levels (measured by MFI; Figure 5.3d; $p = 0.50$) to each age-group. These data imply that both young and elderly-derived MoDCs respond similarly to CD40L stimulation in regards to down-regulation of antigen processing machinery,

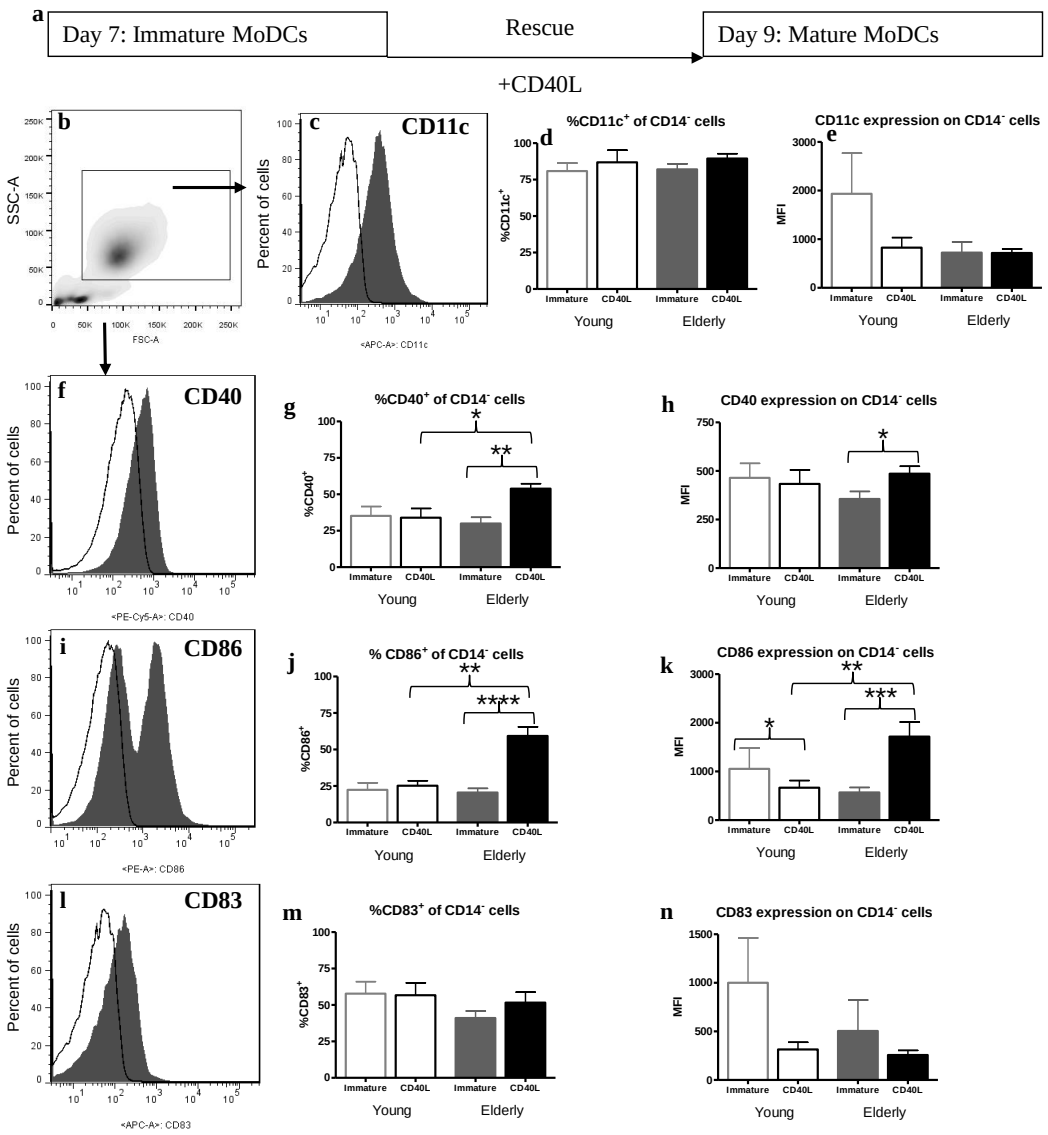

Figure 5.1: CD40 and CD86 expression in elderly CD40L stimulated MoDCs is increased compared to young MoDCs

Immature MoDCs from young and elderly volunteers were stimulated with CD40L (a) and cell surface molecules analysed by flow cytometry. Representative plot (b) showing gating of large cells which were analysed for expression of CD11c (c), CD40 (f), CD86 (i) and CD83 (l) with positive stained cells (grey filled) and unstained cells (unfilled). Pooled data of the percentages of cells positive for CD11c (d), CD40 (g), CD86 (j) and CD83 (m) for immature and CD40L stimulated. Surface expression levels were measured and shown as MFIs of CD11c (e), CD40 (h), CD86 (k) and CD83 (n) in young (n = 10) versus elderly (n = 11) CD40L stimulated MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

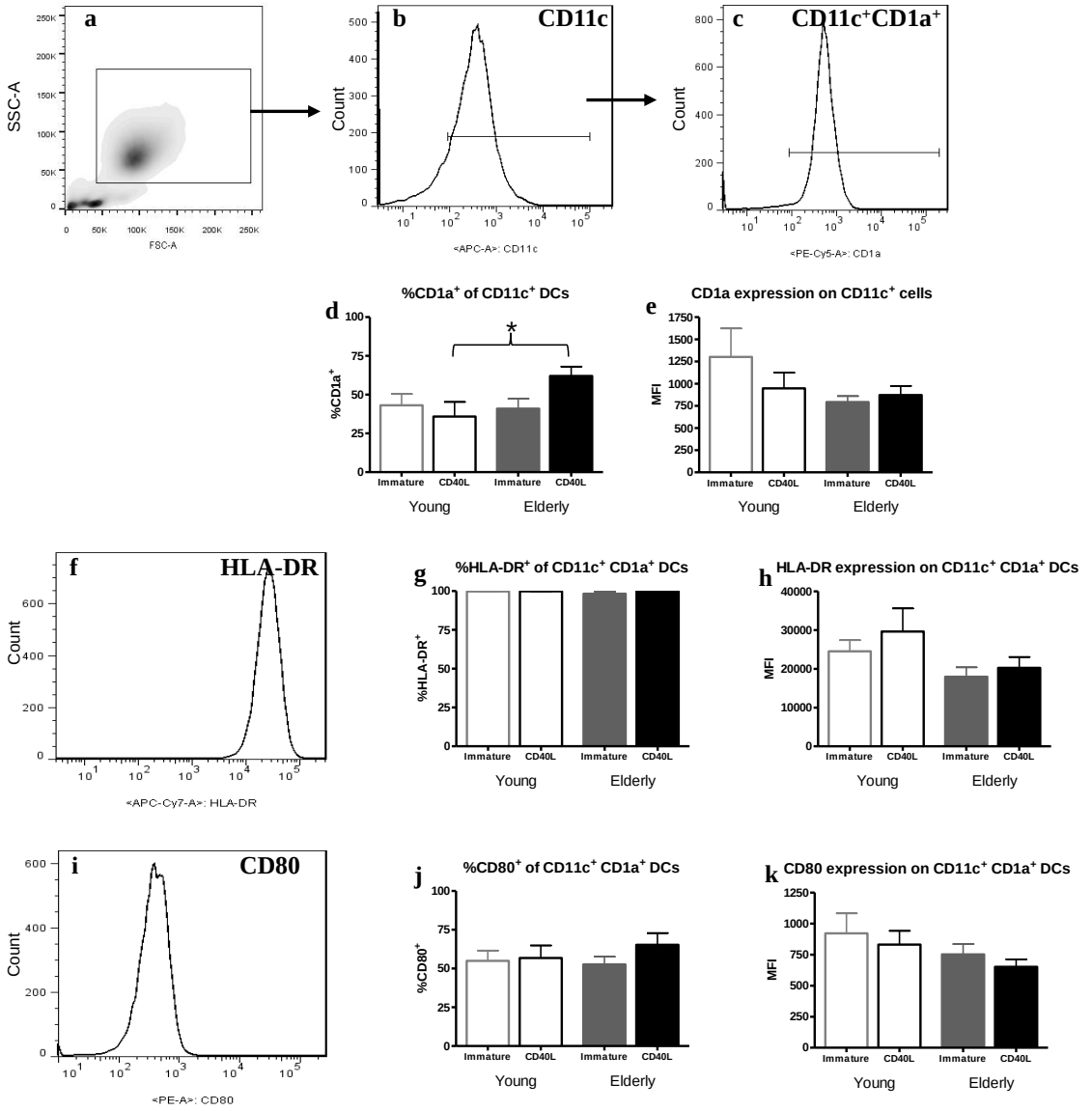

Figure 5.2: The percentage of elderly DCs expressing CD1a is significantly increased following stimulation with CD40L

Immature MoDCs from young and elderly volunteers were stimulated with CD40L and cell surface molecules analysed by flow cytometry. Large cells (a) were identified and gated on $CD11c^+$ cells (b). $CD11c^+$ DCs were further analysed and gated on $CD1a^+$ cells (c). $CD11c^+CD1a^+$ DCs were analysed for expression of HLA-DR (f) and CD80 (i). Pooled data for the percentage of cells positive for CD1a (d), HLA-DR (g) and CD80 (j) and for cell surface expression levels (MFIs) of CD1a (e), HLA-DR (h) and CD80 (k) is from young (n = 10) versus elderly (n = 11) MoDCs (Immature and CD40L stimulated). Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test. *$p<0.05$

and that unlike IFNγ alone, CD40 activation induces full maturation (in terms of loss of antigen presentation) in elderly-derived MoDCs.

5.2.3 Age does not modulate the ability of CD40L-stimulated MoDCs to induce lymphocyte proliferation

In chapter 3, no age-related decrease was observed in the ability of MoDCs to induce T cell proliferation. In fact, elderly derived MoDCs generated a greater proliferative response in young-derived T cells. To investigate whether CD40L stimulation could further improve this response, MoDCs from 6 young and 9 elderly volunteers were incubated with CFSE-labelled lymphocytes from a young universal donor (34 y/o). Lymphocytes were first gated for by size (Figure 5.4a) then by CD4 and CD8 expression (Figure 5.4b), with proliferation of T cells determined by the change in parent population in comparison to unstimulated T cells (Figure 5.4c). Whilst no age related differences were observed in the proliferation of $CD4^+$ T cells (Figure 5.4d), a trend to increased $CD8^+$ T cell proliferation was seen in elderly-derived MoDCs (Figure 5.4e).

5.2.4 Cytokine secretion by CD40L-activated DCs is not affected by age

In chapter 3, studies of cytokine secretion in young and elderly-derived MoDCs, showed an age-relate transition towards pro-inflammatory cytokine production following stimulation with IFNγ. To investigate whether a similar trend occurs after CD40L stimulation, culture media was collected from MoDCs derived from 11 young and 11 elderly healthy individuals and analysed for the secretion of TNF, IL-10, VEGF, IL-12p70 and IFNγ by CBA as described in chapter 3. Production of cytokines by CD40L stimulated MoDCs was very low irrespective of age. Measured concentrations of IL-10, IL-12p70 and IFNγ were below the detection limits of the assay (data not shown). Whilst no difference was observed in the secretion of VEGF (Figure 5.5f; $p = 0.69$) an increased age-related trend was seen in the secretion of TNF (Figure 5.5e; $p = 0.14$).

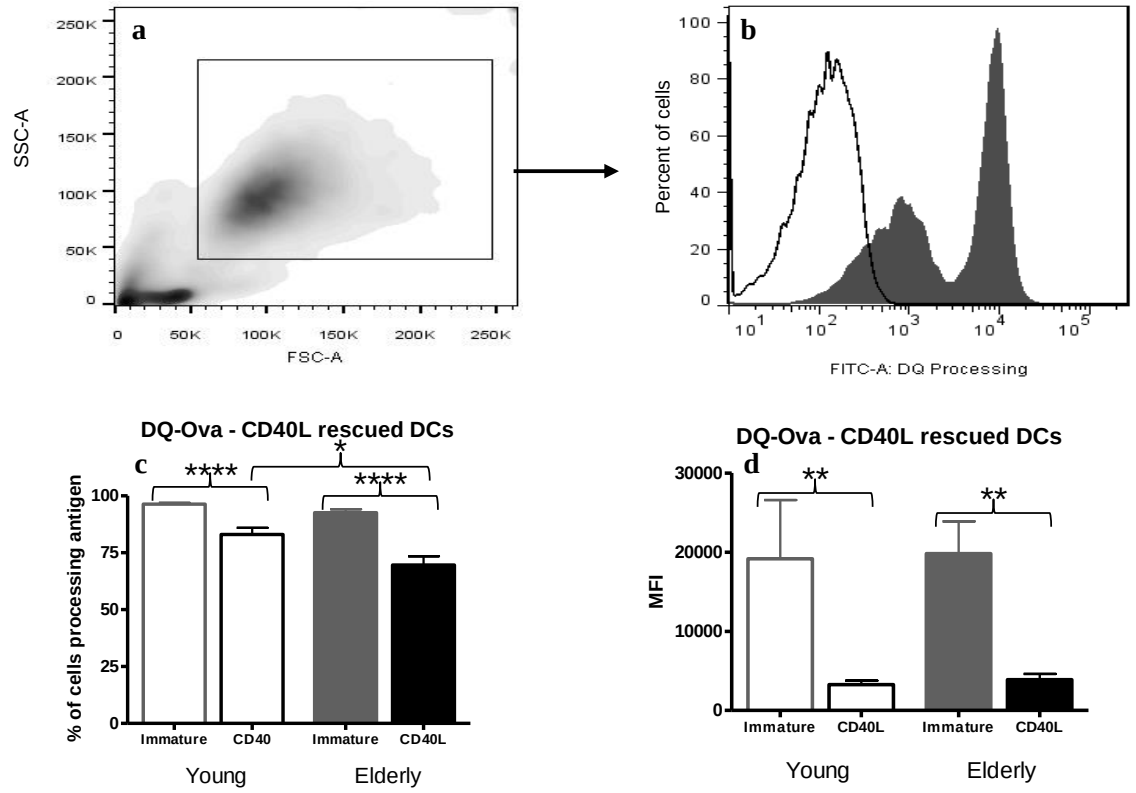

Figure 5.3: Stimulation of elderly-derived MoDCs with CD40L overcomes the antigen processing paralysis observed following LPS +/- IFNγ stimulation

CD40L stimulated MoDCs from young and elderly volunteers were incubated for 1 hour with DQ-Ovalbumin. Representative dot plot (a) showing gating of MoDCs based on size and granularity. The capacity to process antigen was determined by emission of a signal in the FITC channel (b) and measured by flow cytometry; grey histogram represents cells incubated with DQ-OVA, white histogram represents control cells that did not receive DQ-OVA. Pooled data (c) of % of DCs able to process antigen and (d) the mean fluorescent intensity (MFI) indicating relative antigen processing capacity of young (n = 12) versus elderly (n = 12) MoDCs for immature and CD40L stimulated MoDCs. Pooled data is shown as mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001

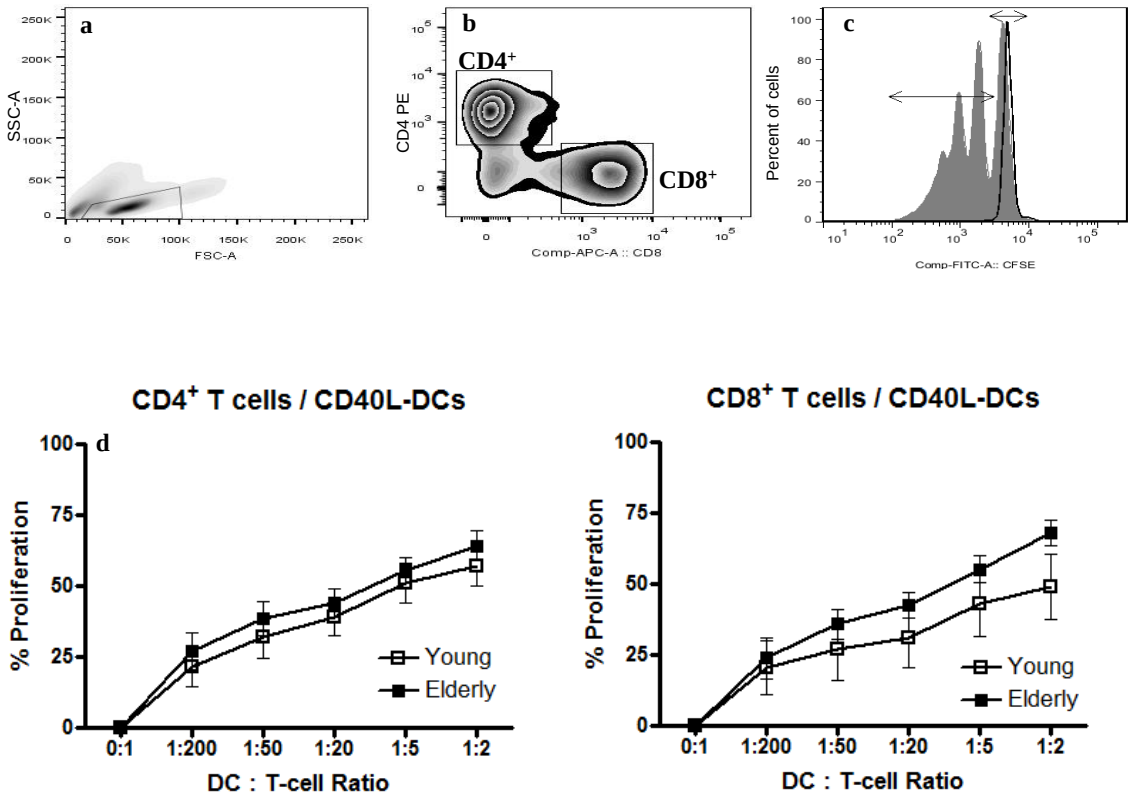

Figure 5.4: Age does not modulate the ability of CD40L-stimulated MoDCs to induce lymphocyte proliferation

CD40L stimulated MoDCs were co-cultured with allogeneic CFSE-labelled lymphocytes for 7 days. Cells were collected and stained for CD4 and CD8 expression and analysed by flow cytometry. Representative plot (a) showing gating of lymphocytes by size and granularity. Lymphocytes were further gated as either $CD4^+$ or $CD8^+$ (b). The percentage of proliferating cells of the total gated population (longer arrow) was determined; the smaller arrow shows the non-proliferating parent peak. Pooled percentage proliferation was plotted against DC:T-cell ratio for $CD4^+$ (d) and $CD8^+$ (e) T-cells in young (n = 6) versus elderly volunteers (n = 9) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test.

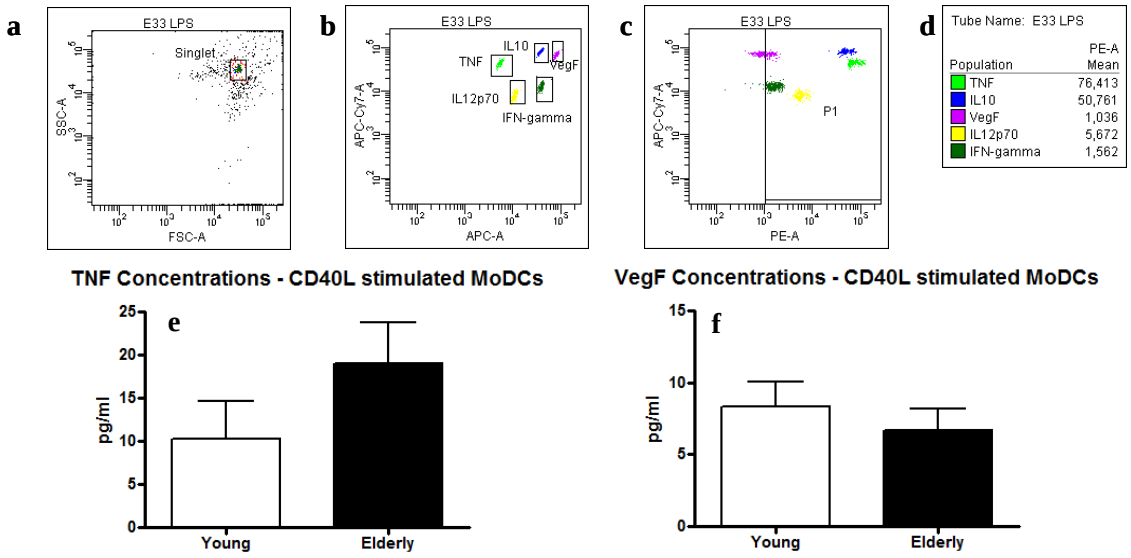

Figure 5.5: Cytokine secretion by CD40L-activated DCs is not affected by age

Following 48 hours stimulation with CD40L, culture media from healthy young and elderly derived MoDCs were analysed for the production of cytokines by cytometric bead array. Representative plot (a) showing gating of pooled beads by size and granularity. Beads were further gated (b) to identify each cytokine tested. Concentration of cytokine present was determine by measuring MFI (c,d) and correlating it to a standard curve. Pooled data for the concentration of TNF (e) and VEGF (f) present in culture media containing young (n = 11) and elderly (n = 11) MoDCs is shown as mean ± SEM.

5.3 Discussion

Age-related changes in dendritic cell function have been implicated as one component of the immune response influencing T cell dysfunction in the elderly (Naylor et al., 2005). The data from chapter 3 highlighted several age-related changes in DC numbers and function. Furthermore, whilst elderly-derived monocytes maintained their ability to differentiate into competent MoDCs, age-related differences became apparent following stimulation with LPS and/or IFNγ. The one function of mature MoDCs that did not appear to be affected by age was their ability to induce proliferation of young-derived T cells. One hypothesis is that young-derived T cells provide a strong CD40L signal to overcome DC dysfunction in the elderly.

As previously mentioned, the interaction between CD40 on DCs and CD154 (CD40L) on T cells induces increased survival in DCs, and up-regulation of costimulatory and MHC molecules, as well as an increase in cytokine secretion (Caux et al., 1994, Koch et al., 1996). Studies have also shown that in the absence of $CD4^+$ T cell help, agonist αCD40 antibody 'licenses' DCs to generate an immune response strong enough to eradicate tumour cells (Bennett et al., 1998, Fransen et al., 2011, Schoenberger et al., 1998, Toes et al., 1998). Therefore, the aim of these experiments was to determine if stimulation of MoDCs with CD40L overcomes elderly-derived MoDC dysfunction.

The studies in chapter 3 showed that immature elderly-derived MoDCs have a trend towards decreased expression of molecules such as CD1a, CD11c, CD40, CD80, CD83, CD86 and HLA-DR. The studies in this chapter clearly show that elderly-derived MoDCs respond better to CD40L stimulation than young-derived MoDCs in terms of increased expression of key molecules involved in antigen presentation, the primary role of mature DCs. Whilst the percentage of young-derived MoDCs expressing CD40, CD80, CD83 and CD86 do not change following CD40L stimulation, the percentage of elderly-derived MoDCs expressing these molecule increases (significantly in the case of CD40 and CD86); these data imply improved co-stimulation in the induction of $CD8^+$ T cells. Furthermore, whilst the percentage of young-derived MoDCs expressing CD1a decreases following stimulation, the percentage increases for elderly-derived MoDCs;

these data imply improved presentation of lipid antigens. This difference is not only limited to the percentage of cells, the relative surface expression (MFI) of CD1a, CD40 and CD86 also increases on elderly-derived MoDCs, whilst decreasing for young-derived MoDCs. In comparison, the studies performed in chapter 3 showed that following stimulation with LPS or IFNγ, elderly-derived MoDCs had decreased expression of these key surface molecules relative to young-derived MoDCs. Only the combination of LPS and IFNγ appeared to provide a strong enough signal to overcome these phenotypic differences seen in the elderly-derived MoDCs. Taken together these data show that whilst responses to stimuli such as LPS and IFNγ decrease with age, CD40 activation overcomes some of these DC defects.

As previously mentioned, young-derived MoDCs down-regulate antigen processing machinery during maturation. The work in chapter 3 showed that the level of decreasing antigen processing (measured using MFI) did not always correlate with the percentage of cells able to process antigen. This was clearly seen with LPS stimulation decreasing both the percentage and capacity (MFI) of antigen processing; in contrast IFNγ-stimulation strongly decreased antigen processing capacity, yet the percentage of MoDCs processing antigen remained >70%. The data showed that whilst young-derived MoDCs responded to either LPS and IFNγ by fully down-regulating antigen processing, elderly derived MoDCs did not fully lose their antigen processing suggesting that a form of maturation paralysis was induced leading to a semi-mature state. Following CD40-stimulation, the young-derived MoDCs generated a similar response to the IFNγ-stimulated young-derived MoDCs, in that a high percentage of MoDCs maintained their processing ability, but the capacity to process antigen (MFI) dropped considerably. In contrast, where the IFNγ-stimulated elderly-derived MoDCs exhibited a paralysis in both the percentage of MoDCs still able to process antigen and the amount of antigen processed, the CD40-stimulated elderly-derived MoDCs responded even better than the young-derived MoDCs. This suggests that CD40L stimulation overcomes the semi-mature state that was induced by LPS and/or IFNγ in elderly-derived MoDCs.

As mentioned previously, the one function of MoDCs that was not affected by age was their ability to induce T cell proliferation. Indeed, elderly-derived MoDCs appeared to have a greater capacity to induce T cell proliferation, irrespective of using LPS or IFNγ stimulation (Chapter 3). The present study further emphasized these results, with elderly-derived CD40-stimulated MoDCs again inducing slightly higher T cell proliferation than young-derived MoDCs. The lack of a further increase in T cell proliferation by CD40L alone could be attributed to the presence of CD40L on the T cells themselves. To investigate this further, both MoDC populations could be assessed in their ability to induce proliferation of elderly-derived T cells which typically have lower expression of CD40L (Eaton et al., 2004, Haynes and Maue, 2009).

In response to activation, MoDCs secrete various cytokines to elicit a TH_1/TH_2 response. The studies from chapter 3 observed that use of IFNγ as a maturation stimulus induced a transition towards a pro-inflammatory DC in elderly-derived MoDCs, as evidenced by increased production of TNF and IL-12p70, with decreased production of IL-10. Following stimulation with CD40L, only TNF secretion increased, and neither IL-10 nor IL-12p70 were secreted. The lack of IL-12p70 production could be attributed to several different reasons. Mosca et al (2000) observed that CD40L alone generated only small amounts of IL-12, however combination of CD40L with IFNγ yielded significantly higher amounts. Alternatively, it may be that only specific cytokines are produced with CD40L stimulation alone as Snijders et al (1998) observed high levels of the subunit IL-12p40 following stimulation with CD40L, yet IL-12p70 was undetectable. Nonetheless, increased secretion of TNF could assist transition towards a pro-inflammatory DC which in turn assists in the clearance of infection in the elderly.

In conclusion, this study showed that the previously observed defects in elderly-derived MoDCs, such as decreased maturation markers and antigen processing ability after LPS/IFNγ stimulation could be ‘rescued’ through stimulation with CD40L to levels that surpasses that seen in young-derived MoDCs. It has been well characterised that T cells from elderly individuals express decreased levels of CD40L compared with those from younger individuals (Eaton et al., 2004). It is also known that the interaction between

CD40L on the T cell and CD40 on the DC is crucial for the generation of a sufficiently strong immune response (Bennett et al., 1998, Clarke, 2000, Schoenberger et al., 1998). Therefore it could be speculated that DCs compensate for the decrease in CD40L expression on T cells by increasing the DCs response to CD40L. Therefore, the use of exogenous CD40L would result in the increased responses shown in this study. As the incidence of cancers such as mesothelioma increased with age, the next chapter determines whether CD40 activation could also be effective in mesothelioma patient-derived MoDCs.

6 CAN CD40 STIMULATION RESCUE MESOTHELIOMA-INDUCED DC DYSFUNCTION?

6.1 Introduction

The studies in chapter 4 showed that mesothelioma patients have significantly reduced numbers of blood pDCs, mDC1s and mDC2s and that whilst MoDCs could be generated from blood monocytes they expressed lower levels of co-stimulatory molecules and demonstrated a significantly reduced ability to process antigen compared with age-matched healthy controls. These MoDCs partially matured in response to LPS and/or IFNγ, which may be an effect of age rather than the presence of mesothelioma

As previously mentioned, the transmembrane glycoprotein CD40 is expressed on many different immune cells, such as T cells, B cells, macrophages and DCs (Grewal and Flavell, 1996, Stout and Suttles, 1996, Van Kooten and Banchereau, 1996). Its activation of DCs (typically through interaction with CD40L on T cells) has been shown to induce up-regulation of co-stimulatory and MHC molecules, increased cytokine production and increased survival. Furthermore, CD40 activation by exogenous CD40L has been shown to 'rescue' DCs from the suppressive effect of tumours (Esche et al., 1999, Hermans et al., 1999, Pinzon-Charry et al., 2006). This led to studies examining whether activating CD40 on DCs could be used as an immunotherapy against cancers.

Preclinical murine studies have investigated whether activating CD40 in vivo leads to an improved anti-mesothelioma immune response. Jackaman et al (2012a) observed complete regression of small mesothelioma tumours and the generation of immune memory following intra-tumoural treatment with an agonist anti-CD40 monoclonal antibody. Likewise, Friendlander et al (2003) observed tumour regression, the generation of anti-tumour CTLs and memory following injection of a plasmid encoding CD40L.

Whilst Chapter 5 showed that CD40 stimulation via CD40L can restore age-related defects in MoDCs, this chapter aimed to determine if CD40 stimulation could also restore the more significant defects seen in mesothelioma patient-derived MoDCs.

6.2 Results

6.2.1 CD40L stimulation does not fully rescue CD1a, CD40, CD83, CD86 and HLA-DR expression in mesothelioma-derived MoDCs

To investigate whether CD40L stimulation 'rescues' DCs from mesothelioma patients, MoDCs from patients and healthy controls (see Table 4.1) were stimulated with CD40L for 48 hours (Figure 6.1a). As shown in previous chapters, cells were gated firstly by size, and then by the absence of CD14 before being investigated for expression of CD11c, CD40, CD83 and CD86. $CD11c^+$ DCs were further investigated for expression of CD1a, CD80 and HLA-DR.

In chapter 4, iMoDCs from people with mesothelioma were shown to have a decreased trend in the percentage of $CD14^-$ cells expressing CD40 or CD83 as well as decreased trends in surface expression levels (MFI) of CD11c, CD80, CD83, CD86 and HLA-DR. In addition people with mesothelioma were shown to have significantly decreased expression of CD40 on their iMoDCs. The data from chapter 4 is repeated in this chapter to allow for comparision following stimulation with CD40L.

CD40L stimulation did not induce changes relative to their immature DC counterpart (data from chapter 4) in terms of the percentage of $CD14^-$ cells expressing CD11c (Figure 6.1b), CD83 (Figure 6.1d), or in their expression levels (Figures 6.1c and 6.1e) in healthy or mesothelioma patient-derived MoDCs. However, CD40L stimulation did not restore the percentage of $CD14^-$ cells expressing CD83 to levels observed in healthy age-matched controls (Figure 6.1d: $p = 0.04$). A significant increase was observed in both the percentage of $CD14^-$ cells expressing CD40 (Figure 6.1f: $p = 0.001$) and CD40 MFI (Figure 6.1g: $p = 0.02$) on healthy-derived MoDCs versus their iMoDCs. This response was not reproduced in mesothelioma patient-derived MoDCs and again there was no evidence of the restoration of CD40 to healthy control levels (Figure 6.1f: $p = 0.002$; Figure 6.1g: $p = 0.0008$). In contrast, the percentage of immature $CD14^-$ cells expressing CD86 significantly increased for both healthy-derived MoDCs (Figure 6.1h: $p < 0.0001$) and mesothelioma patient-derived MoDCs (Figure 6.1h: $p < 0.0001$) after CD40L stimulation. CD86 expression levels also significantly increased for healthy

(Figure 6.1i: $p = 0.0002$) and patient (Figure 6.1i: $p < 0.0001$) MoDCs, nonetheless CD86 expression remained lower on CD40L-stimulated patient relative to healthy MoDCs (Figure 6.1h: $p = 0.003$; Figure 6.1i: $p = 0.011$).

The percentage of $CD11c^+$ DCs expressing CD1a appeared to increase on CD40L-stimulated healthy-derived MoDCs but not for CD40L-stimulated mesothelioma patient-derived MoDCs and CD1a expression levels did not change (Figures 6.2a, $p = 0.01$, and 6b respectively). No changes were observed for the percentage of $CD11c^+CD1a^+$ DCs expressing HLA-DR or CD80, or HLA-DR and CD80 expression levels, for CD40L-stimulated MoDCs from healthy volunteers or mesothelioma patients, restoration of these markers to healthy levels did not occur (Figures 6.2c, 6.2d; $p = 0.046$, 6.2e and 6.2f). Taken together these data imply that there is an impairment in the response of mesothelioma patient-derived MoDCs to activation through CD40.

6.2.2 CD40 activation induces down-regulation of antigen processing in mesothelioma-derived MoDCs

The DQ assay was used to compare the antigen processing capacity of CD40L-stimulated MoDCs from 41 mesothelioma patients and 12 healthy age matched controls. Following CD40 activation, a significant percentage of iMoDCs lost their antigen processing capacity for both age-matched healthy controls (Figure 6.3a: $p < 0.0001$) and mesothelioma patients (Figure 6.3a: $p < 0.0001$); no differences were seen between the healthy and mesothelioma cohorts. Although the proportion of age-matched healthy-derived MoDCs losing antigen processing capacity (24.8%) was higher than for the mesothelioma patient-derived MoDCs (17.8%). In contrast, whilst immature MoDCs from healthy volunteers demonstrated potent antigen processing capacity (measured by MFI), those from mesothelioma patients had lost this function. Nonetheless, CD40L stimulation significantly reduced antigen processing capacity for iMoDCs from healthy (Figure 6.3b; $p = 0.0009$) and mesothelioma patients (Figure 6.3b; $p = 0.023$) relative to their immature MoDC levels. The data suggests that although the antigen processing ability of mesothelioma patient-derived iMoDCs is impaired they do have a limited response to stimulation with CD40L.

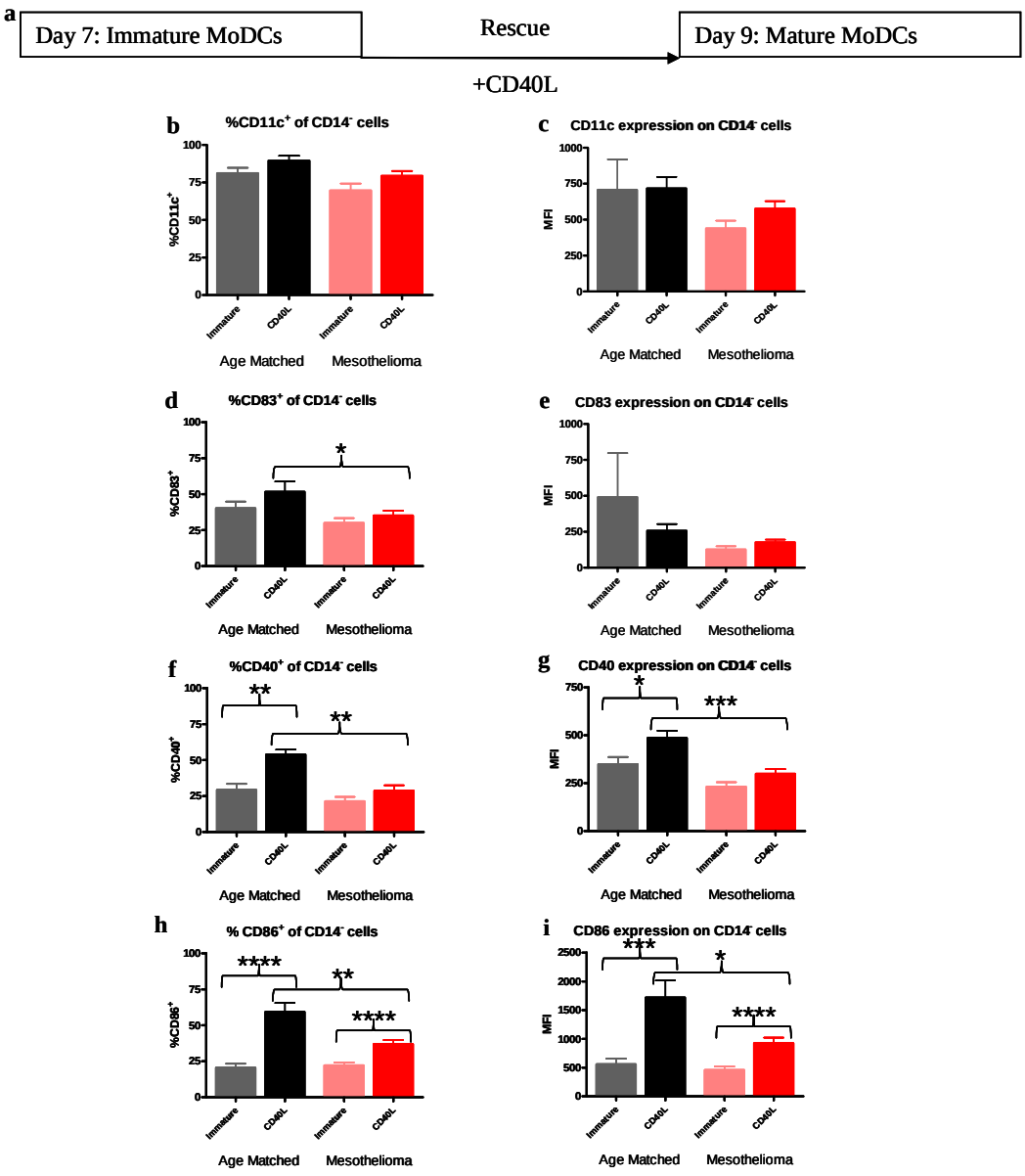

Figure 6.1: CD40, CD86 and CD83 expression in mesothelioma patients CD40-stimulated MODCs is decreased compared to healthy age matched controls

Immature MoDCs generated from mesothelioma patients and age matched volunteers were stimulated with CD40L (a) and cell surface molecules were analysed by flow cytometry. CD14- cells were analysed for expression of CD11c (b,c), CD40 (d,e), CD86 (f,g) and CD83 (h,i). Pooled data of the percentages of cells positive for CD11c (b), CD40 (d), CD86 (f) and CD83 (h). Surface expression levels were measured and shown as MFIs of CD11c (c), CD40 (e), CD86 (g) and CD83 (i) in mesothelioma patients (n = 41) versus age matched volunteers (n = 11) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

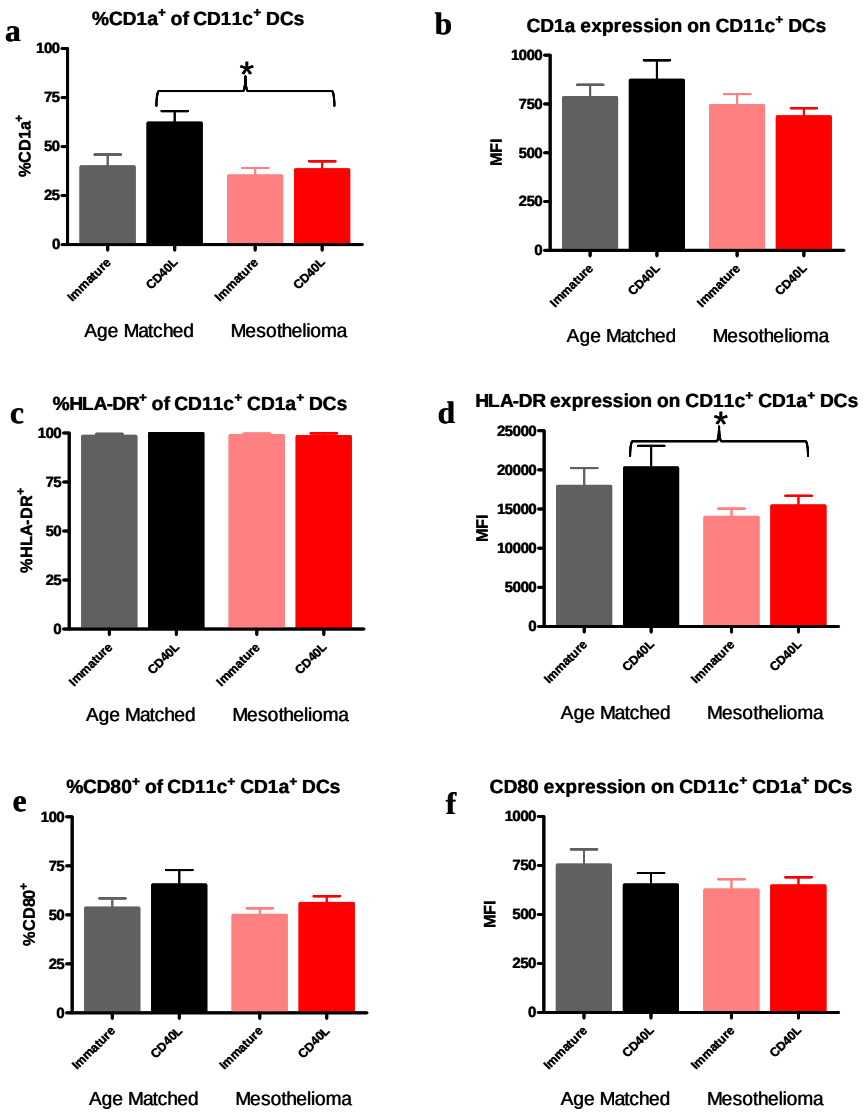

Figure 6.2: The percentage of cells expressing CD1a and the expression of HLA-DR in mesothelioma patients CD40L-stimulated MODCs is reduced compared to healthy controls

Immature MoDCs from mesothelioma patients and age matched volunteers were stimulated with CD40L and cell surface molecules analysed by flow cytometry after gating on $CD11c^+$ cells. $CD11c^+$ DCs were further analysed and gated on $CD1a^+$ cells (a and b). $CD11c^+CD1a^+$ DCs were analysed for expression of HLA-DR (c and d) and CD80 (e and f). Pooled data for the percentage of cells positive for CD1a (a), HLA-DR (c) and CD80 (e) and for cell surface expression levels (MFIs) of CD1a (b), HLA-DR (d) and CD80 (f) in mesothelioma patients (n = 41) versus age matched controls (n = 11) MoDCs. Pooled data is shown as mean ± SEM. P-values were determined using two-tailed Mann-Whitney test. *$p<0.05$

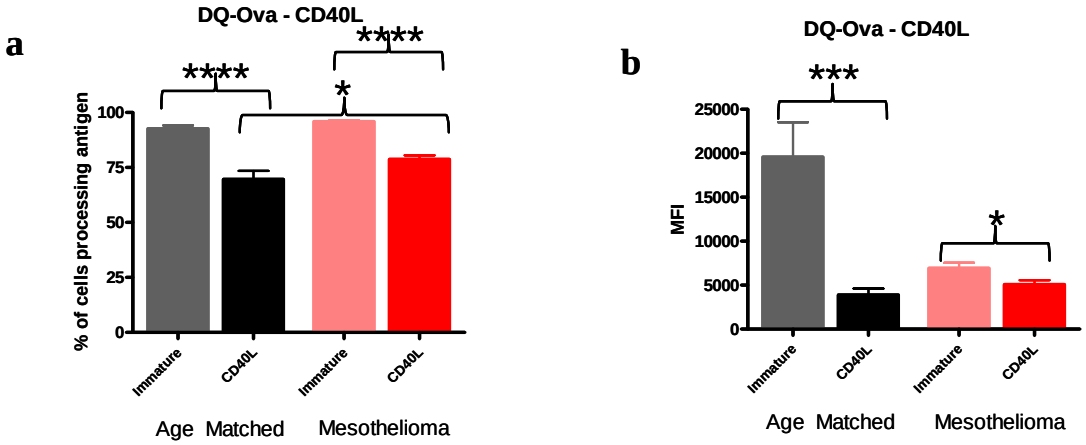

Figure 6.3: MoDCs from mesothelioma patients are impaired in their ability to down-regulate antigen processing following CD40L stimulation

MoDCs from mesothelioma patients and age matched controls previously stimulated with CD40L were incubated for 1 hour with DQ-Ovalbumin. The capacity to process antigen was measured by flow cytometric analysis. Pooled data of the percentage of DCs still able to process antigen (a) and MFIs indicating relative antigen processing capacity of mesothelioma (n = 41) versus age matched (n = 12) MoDCs (Immature versus CD40L stimulated). P-values were determined using the two-tailed Mann-Whitney test. *p<0.05, ***p<0.001, ****p<0.0001

6.2.3 Derivation from patients with Mesothelioma does not affect the ability of CD40L-stimulated MoDCs to induce proliferation of healthy donor T cells

The data from chapter 4 showed no difference in the ability of MoDCs from either mesothelioma patients or healthy controls to induce T cell proliferation. To investigate if there is a change following CD40L activation, MoDCs from 16 mesothelioma patients and 9 age-matched controls were incubated with CFSE-labelled lymphocytes from a young universal donor (34 years old). Proliferation of $CD4^+$ and $CD8^+$ T cells was determined as shown in previous chapters. Regardless of the stimulus used (LPS +/- IFNγ), there was no difference between the ability of age-matched or mesothelioma patient-derived CD40-activated MoDCs to induce $CD4^+$ (Figure 6.4a) or $CD8^+$ (Figure 6.4b) T cell proliferation.

6.2.4 CD40-activated MoDCs from mesothelioma patients or age-matched controls secrete equal concentration of TNF and VEGF

To investigate whether CD40L rescues DC cytokine secretion, culture media collected from MoDCs derived from 44 mesothelioma patients and 11 healthy age-matched individuals was analysed for secretion of TNF, IL-10, VEGF, IL-12p70 and IFNγ by CBA, as described in previous chapters.

Similar to data shown in chapter 5, CD40L stimulation induced only a weak cytokine response. IL-10, IL-12p70 and IFNγ concentrations were below the minimum detection limits of the assay (data not shown). TNF and VEGF were low, but within the minimum detection limits. No differences were observed for TNF (Figure 6.5a: $p = 0.17$) or VEGF (Figure 6.5b: $p = 0.90$) by CD40-activated MoDCs from age-matched controls or mesothelioma patients

6.2.5 Mesothelioma tumour derived factors modulate expression of cell surface molecules after CD40L-stimulation

To investigate whether mesothelioma tumour-derived factors modulate DC function, MoDCs from 5 healthy young adult individuals were cultured in the presence or absence

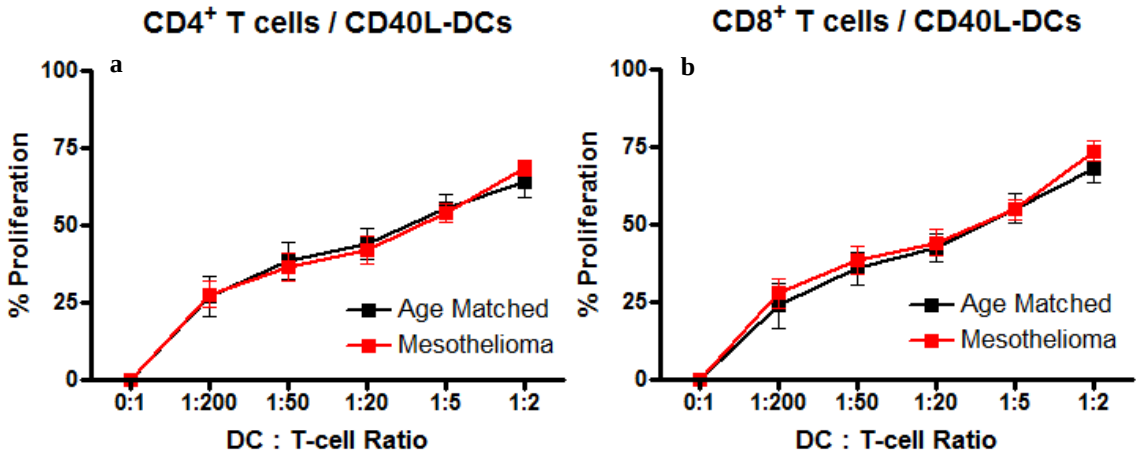

Figure 6.4: CD40L stimulated MoDCs from mesothelioma patients retain their ability to induce T cell proliferation

CD40L-stimulated MoDCs were co-cultured with allogeneic CFSE labelled lymphocytes for 7 days. Cells were collected, stained for CD4 and CD8 expression and analysed by flow cytometry. Pooled percentage proliferation was plotted against DC:T cell ratio for $CD4^+$ (a) and $CD8^+$ (b) T cells in mesothelioma patients (n = 16) versus age match controls (n = 9). Pooled data is shown as mean ± SEM. All P-values were determined using the two-tailed Mann-Whitney test.

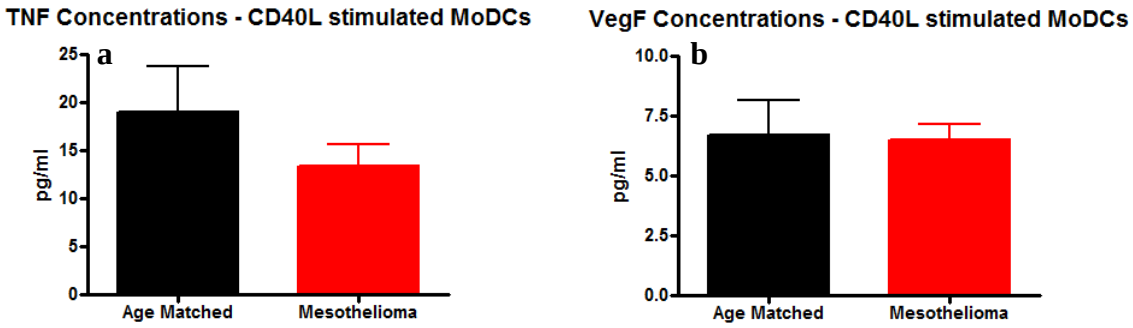

Figure 6.5: CD40L-stimulated MoDCs from mesothelioma patients secrete equal levels of cytokines compared with healthy age matched controls

MoDCs from mesothelioma patients and healthy age matched controls were stimulated with CD40L for 48 hours. Culture media were analysed for the production of cytokines by cytometric bead array. Cytokine concentration was determined by measuring the MFI of the corresponding beads as per figure 5. Pooled data for the concentration of TNF (a) and VEGF (b) secreted by MoDCs from mesothelioma patients (n = 44) and age matched controls (n = 11). Pooled data is shown as mean ± SEM.

of conditioned media collected from two human mesothelioma cells lines, JU77 and ONE58. Stimulation with LPS was used as a positive control. Following incubation, immature MoDCs were stained and analysed by flow cytometry, as described above.

Exposure of healthy iMoDCs to mesothelioma tumour supernatant did not change expression levels of CD40, CD83 and HLA-DR (Figures 6.6b, 6.6c and 6.7c). Significant increases were observed in expression of CD11c and CD86 after exposure to ONE58, but not JU77 (Figures 6.6a and d). CD80 significantly increased in response to JU77 and ONE58 (Figure 6.7b). In contrast, CD1a expression significantly decreased after exposure to JU77 (Figure 6.7a); see Table 6.1.

Table 6.1: Statistical analysis of data from immature DCs from Figures 6.6 and 6.7

	JU77 SN	ONE58 SN
CD1a MFI	**p = 0.044**	p = 0.16
CD11c MFI	p = 0.303	**p = 0.014**
CD40 MFI	p = 0.99	p = 0.26
CD80 MFI	**p = 0.0084**	**p = 0.01**
CD83 MFI	p = 0.81	p = 0.23
CD86 MFI	p = 0.18	**p = 0.032**
HLA-DR MFI	p = 0.26	p = 0.16

LPS stimulation of healthy control MoDCs induced significant increases in CD40 (p = 0.02), CD86 (p = 0.0002), CD80 (p = 0.0004) and HLA-DR (p = 0.007) relative to their immature MoDC counterparts (Figures 6.6b, 6.6d, 6.7b and 6.7c). In contrast, no changes were seen following LPS stimulation in CD11c, CD1a, CD40, CD80, CD83, CD86 and HLA-DR expression in iMoDCs exposed to either tumour supernatant. Tumour-exposed LPS-activated MoDCs demonstrated significantly lower expression of CD80 and CD86 in comparison to LPS-activated healthy MoDCs (Figures 6.7b and 6.6d) irrespective of the supernatant used, see Table 6.2.

Table 6.2: Statistical analysis of data from LPS matured DCs from Figures 6.6 and 6.7

	JU77 SN	ONE58 SN
CD1a MFI	p = 0.061	p = 0.058
CD11c MFI	p = 0.12	**p = 0.022**
CD40 MFI	**p = 0.005**	**p = 0.004**
CD80 MFI	p = 0.05	**p = 0.023**
CD83 MFI	p = 0.17	p = 0.11
CD86 MFI	**p = 0.0007**	**p = 0.0004**
HLA-DR MFI	p = 0.16	p = 0.17

Stimulation of healthy MoDCs with CD40L generated a much weaker response than LPS, with no significant changes observed in expression of any markers when MoDCs were stimulated with either 0.66 µg/ml (1x) or 3.3 µg/ml (5x) CD40L. At the highest concentration tested, 16.5 µg/ml (25x), CD40L-stimulation induced slight but significant increases in CD80 (Figure 6.7b: p = 0.008) and CD86 (Figure 6.6d: p = 0.043) from levels observed on immature MoDCs.

Similar to LPS-activation, CD40L-activation induced a decrease in CD11c expression (Figure 6.6a) on the tumour-exposed MoDCS. CD1a (already reduced by tumour supernatant; Figure 6.7a), CD40 (Figure 6.6b) and HLA-DR (Figure 6.7c) were not affected in comparison to iMoDCs irrespective of the concentration of CD40L used. In contrast, CD40L-stimulated MoDCs generated in both tumour-derived factors further increased CD80 (Figure 6.7b) and CD86 (Figure 6.6d) relative to CD40L-stimulated healthy controls and to tumour-exposed iMoDC controls, Table 6.3.

Table 6.3: Statistical analysis of data from CD40L stimulated DCs from Figures 6.6 and 6.7

	JU77 SN			ONE58 SN		
	0.66 μg/ml	3.3 μg/ml	16.5 μg/ml	0.66 μg/ml	3.3 μg/ml	16.5 μg/ml
CD1a MFI	**p = 0.004**	**p = 0.006**	p = 0.05	**p = 0.005**	**p = 0.006**	**p = 0.04**
CD11c MFI	p = 0.55	p = 0.44	p = 0.75	p = 0.58	p = 0.13	p = 0.11
CD40 MFI	p = 0.63	p = 0.20	p = 0.99	p = 0.80	p = 0.70	p = 0.18
CD80 MFI	**p = 0.002**	**p = 0.002**	**p = 0.04**	**p = 0.0009**	**p = 0.002**	**p = 0.02**
CD83 MFI	p = 0.79	p = 0.63	p = 0.92	p = 0.33	p = 0.29	p = 0.08
CD86 MFI	p = 0.013	p = 0.016	p = 0.08	p = 0.07	p = 0.21	p = 0.47
HLA-DR MFI	**p = 0.02**	**p = 0.02**	p = 0.12	**p = 0.01**	**p = 0.03**	p = 0.11

6.2.6 Patients with a higher percentage of CD80 expressing MoDCs following CD40L stimulation live significantly longer

To investigate whether response to CD40L activation correlates with increased survival, patients were ranked according to their expression (both MFI and %) of cell surface markers, from which a Kaplan-Meier plot was generated. Patients were divided into two groups, those above the median value, and those below. Whilst no significant difference was observed for most responses (data not shown), a significant correlation was observed between a higher than median increase in the percentage of $CD80^+$ MoDCs following CD40L activation and survival time (Figure 6.8).

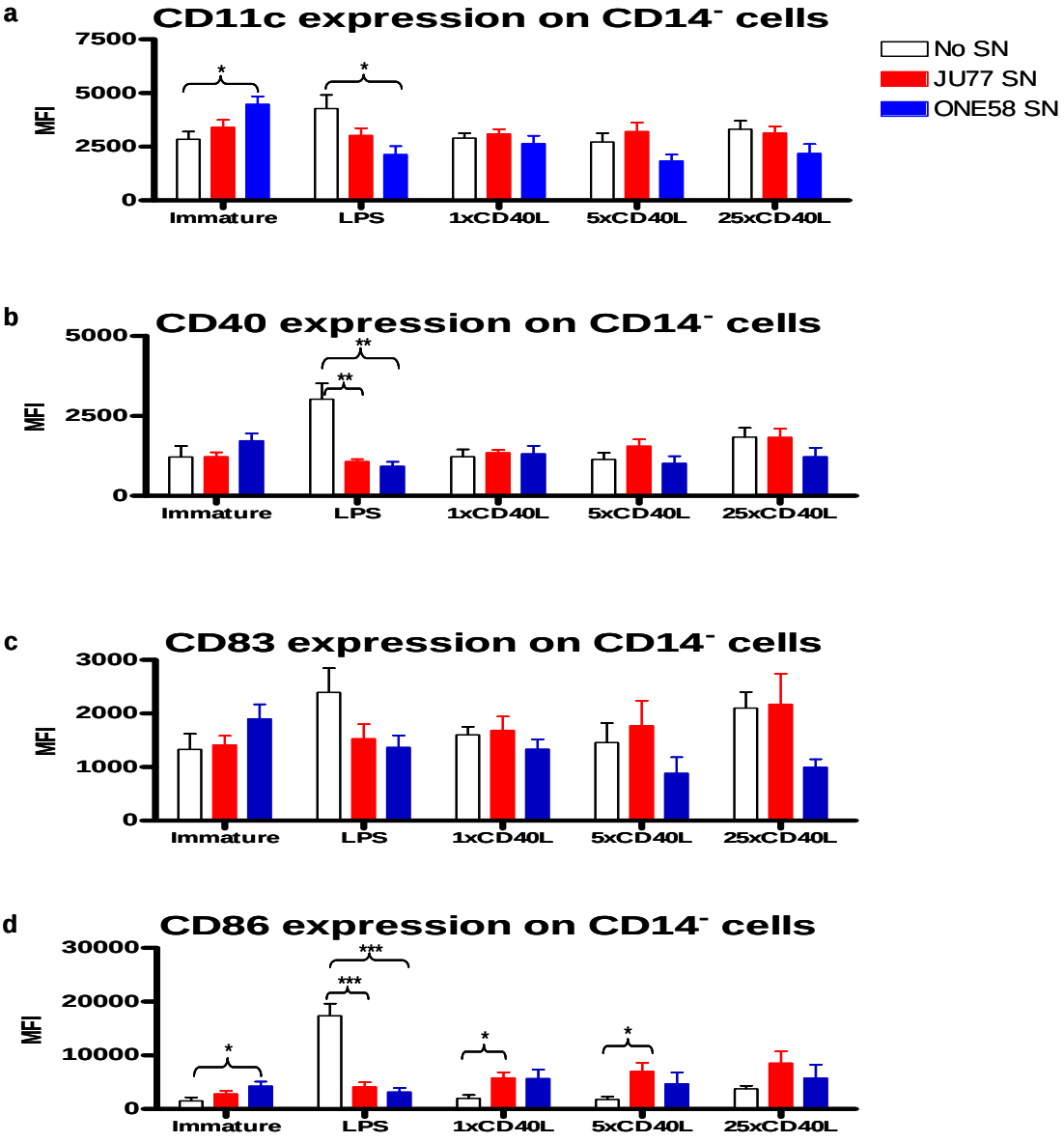

Figure 6.6: Tumour derived factors modulate the expression of CD11c, CD40, CD83 and CD86 before and after stimulation

Immature MoDCs from young healthy volunteers (n = 5) were stimulated with either LPS, or 3 different concentrations of CD40L whilst being cultured in either normal media or media containing 50 % mesothelioma tumour supernatant (JU77 or ONE58). Cells were then analysed for the expression of cell surface molecules by flow cytometry. Surface expression levels were measured and pooled data shown as MFIs for CD11c (a), CD40 (b), CD83 (c) and CD86 (d). Data is shown as mean ± SEM. P-values were determined using Student T test as a comparison to no supernatant (SN). *p<0.05, **p<0.01, ***p<0.001

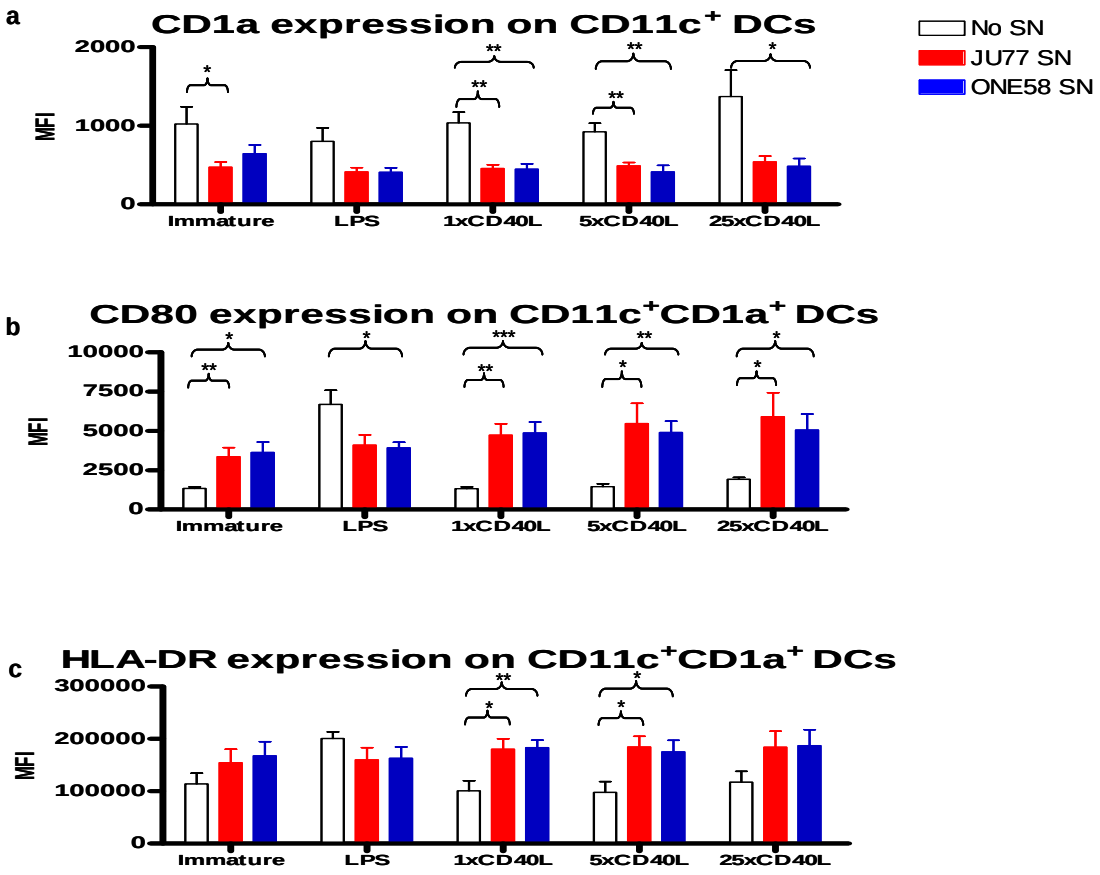

Figure 6.7: Tumour derived factors modulate the expression of CD1a, CD80 and HLA-DR before and after stimulation

Immature MoDCs from young healthy volunteers (n = 5) were stimulated with either LPS, or 3 different concentrations of CD40L whilst being cultured in either normal media or media containing 50 % mesothelioma tumour supernatant (JU77 or ONE58). Cells were then analysed for the expression of cell surface molecules by flow cytometry. Surface expression levels were measured and pooled data shown as MFIs for CD1a (b), CD80 (d) and HLA-DR (f). Data is shown as mean ± SEM. P-values were determined using Student T test as a comparison to no supernatant (SN). *p<0.05, **p<0.01, ***p<0.001

a

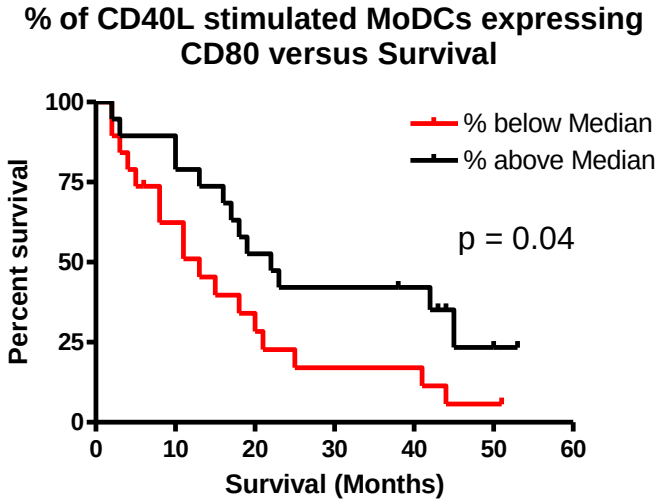

Figure 6.8: Patients with a higher percentage of CD80 expressing MoDCs following CD40L stimulation live significantly longer

Immature MoDCs generated from mesothelioma patients were stimulated with CD40L and the expression of cell surface molecules were analysed by flow cytometry as previously described (Figures 6.1 and 6.2). Patients were ranked according to the percentage of CD40L stimulated MoDCs expressing CD80. Percent survival was then plotted against survival (in months) from time of blood collection (a), with patients dichotomised into those above the median, and those below the median percentage CD80 expression. The p value was determined using the Logrank Test.

6.3 Discussion

Many cancers have been shown to induce defects in DCs. These include decreased numbers of circulating DCs (Almand et al., 2000, Sciarra et al., 2007) and decreased expression of co-stimulatory and MHC molecules (Gabrilovich et al., 1997, Melichar et al., 1998, Sakakura et al., 2006). The results in chapter 4 showed that mesothelioma patients had reduced numbers of circulating DC subsets as well as defects in the ability of monocytes to differentiate into MoDCs; these MoDCs demonstrated a reduced capacity to process antigen and upregulate co-stimulatory molecules in response to LPS and/or IFNγ.

CD40-targetting therapy has been investigated for its potential to activate DCs and generate anti-tumour CTL responses in the absence of $CD4^+$ T cell help (Bennett et al., 1998, Toes et al., 1998). Murine studies have shown regression of mesothelioma tumours following administration of an agonist αCD40 antibody (Jackaman et al., 2011, Stumbles et al., 2004). To date, no study has investigated whether CD40L therapy has the potential to overcome mesothelioma-induced DC dysfunction. Therefore, the aim of this chapter was to determine whether CD40L activation could rescue DCs derived from mesothelioma patients.

The results from chapter 4 showed that mesothelioma patient-derived MoDCs were not equivalent to their healthy counterparts in their immature state (i.e. decreased CD11c, CD40, CD80, CD83, CD86 and HLA-DR) and this status was maintained following maturation with LPS and/or IFNγ. Unlike the results shown in chapter 5, where elderly-derived MoDCs responded better to CD40L activation than young-derived MoDCs, DCs from mesothelioma patients (which are primarily an elderly population) responded very poorly to CD40L activation. The patients' MoDCs exhibited significant defects in the percentage of cells expressing CD1a, CD40, CD83 and CD86, combined with lower expression levels of CD40, CD86 and HLA-DR after CD40L activation. Furthermore, whilst healthy, age-matched derived MoDCs responded to CD40L by significantly increasing the percentage of cells expressing CD40 and CD86, as well as surface expression of CD40 and CD86, patient-derived MoDCs only significantly increased the

percentage and expression of CD86. The increase in CD86 was not sufficient to reach healthy levels. In contrast, Pinzon-Charry et al (2006) showed a significant rescue of MoDCs from breast cancer patients when activated with CD40L (2 μg/ml). It is possible that the concentration used in the study in this chapter (0.66 μg/ml) was too low to induce a sufficient response. Moreover, the patients in the study by Pinzon-Charry had stage I and II breast cancer rather than advanced disease, and their DCs could be more responsive to CD40 activation than those from mesothelioma patients as immunosuppression has been shown to be more profound with more advanced cancer stage (Diaz-Montero et al., 2009, Wang et al., 2013).

Interestingly, increased survival was observed in patients with a higher than median percentage of $CD80^+$ MoDCs following stimulation with CD40L. Whilst this data does not take into regards any therapy the patients may have had post collection, it does suggest a possibly indicator for either increased survival, or possibly a marker for identification of patients who may respond better to CD40 stimulation.

The studies in chapter 4 showed defects in the ability of immature MoDCs from mesothelioma patients to process antigen. Following stimulation with LPS and/or IFNγ, the patient-derived MoDCs reduction in antigen processing was similar to aged-matched healthy-derived but not to younger healthy-derived MoDCs, implying an age-related defect in the maturation process. CD40L also induced a significant decrease in the ability of patient MoDCs to process antigen, again implying maturation to similar levels to the age-matched controls. However, this response might not be useful as patient DCs were incapable of processing antigen in their immature state.

Following CD40L stimulation, patient-derived MoDCs induced the same levels of $CD4^+$ and $CD8^+$ T cell proliferation as their healthy-derived counterparts indicating that mesothelioma has no effect on the allo-antigen presenting capacity of MoDCs. However, an inability to process antigens in mesothelioma patient iMoDCs implies that tumour antigens may not be processed for presentation to T cells.

DCs respond to activation stimuli by secreting cytokines (Shortman and Liu, 2002). Results from chapter 4 showed that the cytokine response by MoDCs from mesothelioma patients is determined by the stimulus used. LPS stimulation generated MoDCs that produced both anti-inflammatory and pro-inflammatory cytokines. IFNγ activation led to a weak cytokine response similar to the healthy controls. Age-matched and mesothelioma patient MoDCs produced very low levels of cytokines in response to CD40L with only VEGF and TNF detected; Mesothelioma MoDCs showed a trend to lower production of TNF. The lack of a strong cytokine response may reflect the weak stimulatory effect of CD40L rather than any impairment in MoDC cytokine production due to mesothelioma.

Tumour cells secrete many soluble factors (Ladisch et al., 1983, Mattei et al., 1994, Moradi et al., 1993, Toi et al., 1996, Zhang and Adachi, 1999), which inhibit various components of DC function (Gabrilovich et al., 1996, Hayashi et al., 2003, Serafini et al., 2004a, Shurin et al., 2001b, Steger et al., 1997, Weber et al., 2005). Healthy-derived monocytes exposed to supernatants collected from two different mesothelioma cell lines revealed changes in expression levels of typical DC surface molecules on immature MoDCs. Exposure to mesothelioma-derived factors induced significantly increased expression of CD11c, CD80 and CD86, yet a significant decrease in CD1a expression on MoDCs. Kiertscher et al (2000) observed similar results when healthy MoDCS were differentiated in the presence of 50% tumour supernatant obtained from the lung carcinoma cell line, A549. In their study, increased expression of CD11c, CD40, CD80, CD83 and CD86 was observed. Likewise, Li et al (2007) observed increased expression of CD40, CD80, CD83, CD86 and HLA-DR on immature MoDCs differentiated in the presence of 30% hepato-carcinoma cell line supernatant. Taken together, these data suggest that tumour-derived factors induce premature maturation in MoDCs, possibly generating tolerogenic DCs to restrict immune responses to the tumour.

Following activation with LPS, MoDCs cultured with mesothelioma tumour-supernatant demonstrated a muted response relative to controls including significant decreases in expression of CD11c, CD40, CD80 and CD86. Similar results were reported by Li et al

(2007) using MoDCs exposed to tumour supernatant collected from hepatoma-derived cells lines. Likewise, Michielsen et al (2011) did not see an increase in CD83, CD86 and HLA-DR expression in LPS-activated MoDCs pre-exposed to colorectal carcinoma supernatant. These data indicate that tumour-derived factors induce maturational paralysis in healthy MoDCs. This suggests that even if patient DCs migrated from the tumour site (and away from tumour-derived factors) to the lymph nodes, they would not be fully matured and would have difficulties in presenting any antigens they may have processed.

CD40L stimulation generated a weak response with only the high concentration (16.5 µg/ml) generating a significant increase in CD80 and CD86 and did not rescue mesothelioma patient MoDCs or MoDCs generated in the presence of tumour-derived factors. These data suggest that mesothelioma tumour-derived factors induce a permanent maturational paralysis that cannot be fully recovered through CD40 activation

In conclusion, these results demonstrate that MoDCs from mesothelioma patients respond poorly to CD40L activation. CD40L-activation did not restore DC phenotype to healthy levels nor did it induce loss of antigen processing capacity as expected upon maturation. Furthermore, exposure of healthy-derived MoDCs to mesothelioma tumour cell supernatants induced lasting defects that could not be reversed by CD40L activation. Therefore, these data suggest that mesothelioma tumours are highly suppressive and that rescue through CD40 might require combination treatment with other modalities.

7 CASE STUDIES

7.1 Introduction

The studies in chapters 4 and 6 showed significant defects in mesothelioma patient DC subsets. Enumeration studies showed significantly decreased numbers of all circulating DC subsets in patients. The generation of MoDCs from patient monocytes revealed impairments in the expression of surface markers, including decreased MHC and co-stimulatory molecules on both immature and activated MoDCs, as well as a decreased ability to process antigen. Furthermore, CD40 stimulation could not rescue the phenotype and function of these MoDCs. Chapter 6 confirmed that mesothelioma tumour-derived factors are likely to play a role in inducing DC dysfunction. Therefore, reducing tumour burden by surgery and/or radiotherapy might restore DC function as the concentrations of tumour-derived factors are also likely to be reduced.

The effect of surgery and/or radiotherapy on DC subsets in other cancers has been studied with contradictory results. Feng et al (2012), Pinzon-Charry et al (2007) and Ma et al (2009) reported decreases in either mDCs alone, or both mDCs and pDCs following surgery for bladder, breast and laryngeal cancers respectively. Studies into the effect of surgery on the phenotype of MoDCs generated from cancer patients also resulted in a variety of results. Feng et al (2012) described a transient decrease in CD80 and CD86 expression immediately after surgery; within seven days their expression returned to pre-surgery levels. In contrast, both Ma et al (2009) and Brusa et al (2011) observed an increase in expression of CD80, CD83, CD86 and HLA-DR after surgery. Similarly, studies on the effect of radiotherapy on DCs have produced conflicting data. Ma et al (2009) observed an increase in the numbers of mDCs post radiotherapy, whilst Pinzon-Charry et al (2007) showed decreasing numbers of circulating mDCs and pDCs.

The studies in this chapter were opportunistic hypothesis-generating studies in small patient subsets undergoing specific procedures as part of routine clinical care. As such, these studies aimed to investigate whether surgery or radiotherapy restore mesothelioma-induced DC dysfunction to healthy levels and to investigate whether DCs

located closer to the tumour site (pleural fluid) are different to those in the circulation. As only small patient numbers underwent each of these procedures, these studies are considered of interest and hypothesis generating but not definitive

7.2 Results

7.2.1 Radiotherapy does not restore blood DC numbers in mesothelioma patients to healthy levels

Three patients enrolled in the study were planned to have palliative local radiotherapy as part of their recommended clinical treatment (Table 7.1). To investigate whether radiotherapy has any effect on circulating DC subset numbers blood was collected in the week prior to radiotherapy commencing and one week following the end of radiotherapy (Figure 7.1a) from three patients and pDCs, mDC1s and mDC2s numbers determined as shown in chapter 3, Figure 1. Similar to the data shown in chapter 4, all three mesothelioma patients had decreased numbers of DC subsets as compared with healthy age-matched controls prior to radiotherapy. Following radiotherapy, all three DC subsets increased in two of the patients, although their numbers did not reach those seen in healthy controls (Figures 7.1b-d). The third patient experienced a decrease in all circulating DC numbers following radiotherapy.

Table 7.1: Dose and number of fractions for radiotherapy patients

Patient ID	Dosage of Radiotherapy	No of Doses
Radiotherapy P1	30 Gy	10
Radiotherapy P2	30 Gy	10
Radiotherapy P3	45 Gy	15

7.2.2 Radiotherapy decreases the antigen processing capacity of mesothelioma patient derived MoDCs

The data in chapter 4 showed that immature MoDCs from mesothelioma patients have decreased capacity to process antigen. Nonetheless they achieved some level of maturation as they down-regulated their antigen processing ability following activation. To determine if radiotherapy restored antigen processing ability to healthy levels, the ability of immature versus LPS, IFNγ, LPS + IFNγ or CD40L matured MoDCs generated from blood collected pre and post radiotherapy to process antigen was measured using the DQ-OVA assay.

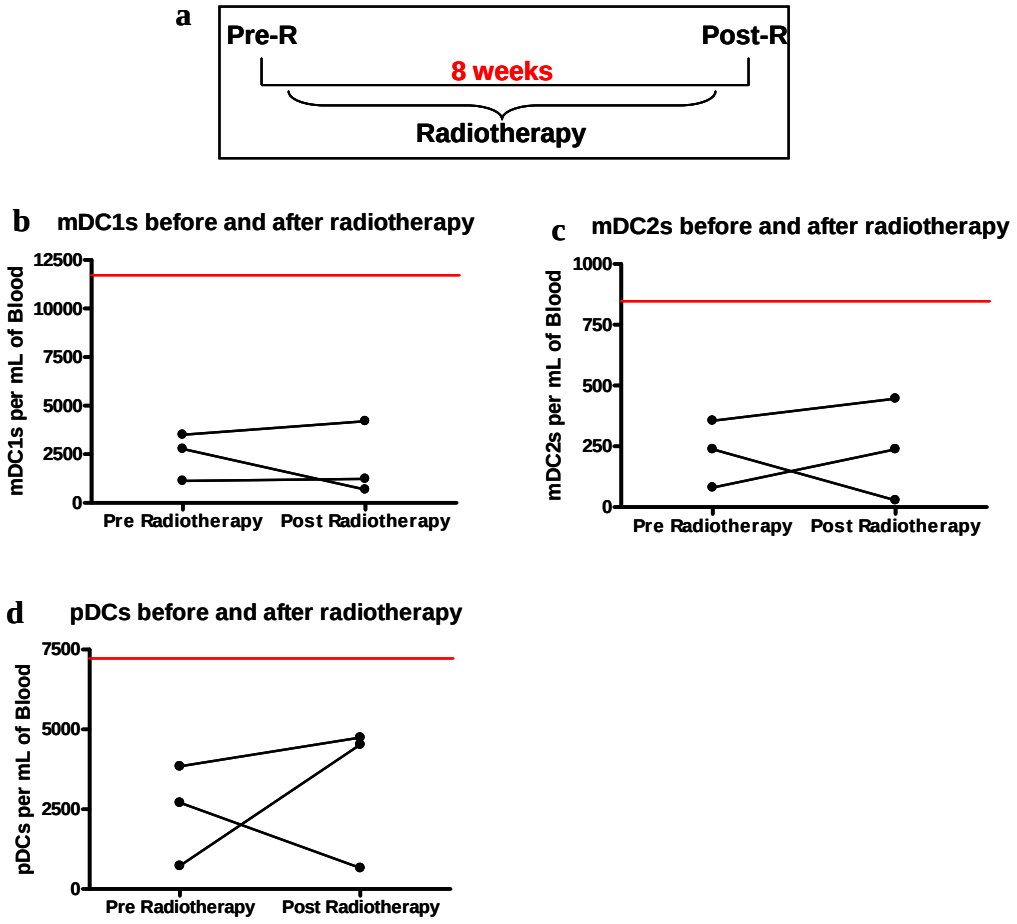

Figure 7.1: Radiotherapy does not restore blood DC numbers in mesothelioma patients to healthy levels

Whole blood collected from mesothelioma patients pre- and post-treatment with radiotherapy was stained for DC subpopulations and analysed by flow cytometry (a). DC subpopulations were identified by high expression of BDCA-1 (b: mDC1), BDCA-3 (c: mDC2) and BDCA2 (d: pDC). Absolute counts were determined by multiplying the number of DCs by the number of PBMCs determined using a haemocytometer. Circulating mDC1, mDC2 and pDCs are shown as the number of DCs per ml of blood. Each joined pair of dots represents an individual volunteer (n = 3). The red line represents DC levels in aged-matched healthy controls.

No difference was observed in the percentage of immature pre- versus post-radiotherapy MoDCs able to process antigen (Figure 7.2a), however despite the small patient numbers a clear trend was seen for a decrease in the level of antigen processing capacity of post radiotherapy MoDCs in comparison to pre radiotherapy MoDCs (Figure 7.2b: $p = 0.09$). This difference was further decreased relative to healthy controls.

Regardless of the stimulus used, the MoDCs generated post-radiotherapy had a reduced ability to process antigen relative to activated MoDCs pre-radiotherapy, with significant decreases seen in the proportion of MoDCs able to process antigen when activated with either LPS ($p = 0.01$) or the LPS/IFNγ combination ($p = 0.001$; Figure 7.2c). It should be noted that the pre-radiotherapy baseline percentage of MoDCs able to process antigen following LPS and LPS/IFNγ activation was higher than that observed in healthy controls. For all stimuli, post-radiotherapy MoDCs exhibited decreased antigen processing levels (MFI) in relation to pre radiotherapy MoDCs. With significant decreased levels observed following activation with LPS ($p = 0.0098$) and LPS/IFNγ ($p = 0.004$; Figure 7.2d). The data suggest that whilst radiotherapy decreases the antigen processing capacity of iMoDCs, possibly via premature maturation, it also improves the expected maturation-associated loss of antigen processing ability post activation, implying restoration of some aspects of DC maturation.

7.2.3 Radiotherapy modulates expression of CD11c, CD40, CD83, CD86 and HLA-DR on mesothelioma patient iMoDCs

To investigate whether radiotherapy rescues DC function, MoDCs were generated from monocyte precursors from the same three patients' blood collected pre and post radiotherapy. Cells were first gated by size, then by the absence of CD14 expression, before being analysed for the expression of CD11c, CD40, CD83 and CD86. $CD11c^+$ DCs were further analysed for the expression of CD1a, CD80 and HLA-DR and results are reported as the percentage of cells expressing each marker, as well as the surface expression levels (MFI).

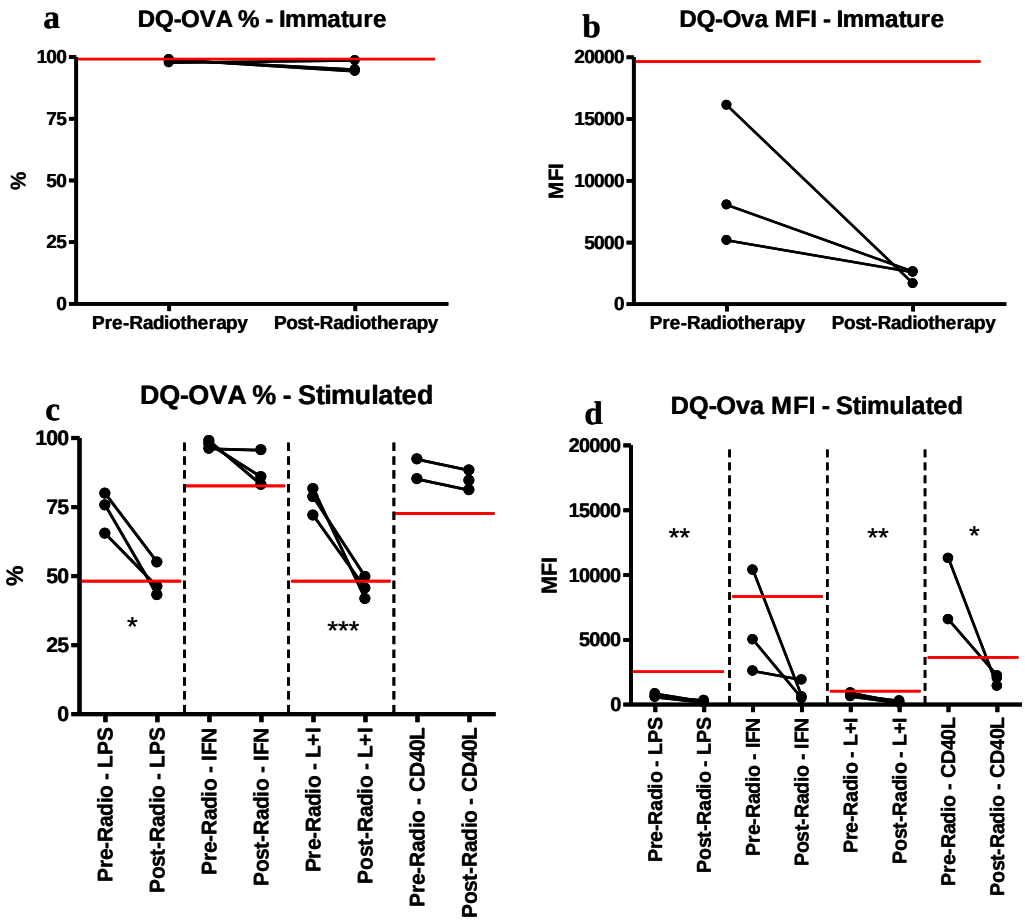

Figure 7.2: The antigen processing capacity of mesothelioma patient MoDCs is decreased following radiotherapy

MoDCs generated from monocytes from mesothelioma patients (pre and post radiotherapy), were incubated for 1 hour with FITC-labeled DQ-Ovalbumin (DQ-OVA). The capacity to process antigen was measured by flow cytometry. Pooled data (a) of the % of DCs able to process antigen and (b) the mean fluorescent intensity (MFI) indicating the relative antigen processing capacity of mesothelioma (n=3) iMoDCs pre and post radiotherapy (n=3). Pooled data (c) shows the % of DCs able to process antigen and the MFI (d) of stimulated MoDCs from mesothelioma patients (n=3) pre and post radiotherapy. Pooled data is shown as mean ± SEM. Average % and MFI of healthy controls is shown as red lines. P values were determined by unpaired T tests. *p<0.05, **p<0.01, ***p<0.001

Two patients showed both an increased percentage of immature $CD14^-$ cells expressing CD40 and increased CD40 surface expression levels (MFI); the third patient showed an increase in CD40 expression whilst the percentage of $CD14^-$ cells expressing CD40 decreased. CD11c, CD83 and CD86 expression decreased following radiotherapy in all three patients with a statistically significant decrease seen in the percentage of immature $CD14^-$ cells expressing CD11c, CD83 or CD86, as well as the surface expression of CD11c and CD83 (Figures 7.3 a-h), see Table 7.2.

CD1a expression increased in $CD11c^+$ DCs in two patients and decreased in the third patient (Figures 7.4a and 7.4b). Similarly, the percentage of $CD11c^+CD1a^+$DCs expressing CD80 increased in two patients and decreased in the third patient, whilst CD80 expression levels increased or did not change in all three patients (Figures 7.4c and 7.4d). There was no change in the percentage of $CD11c^+CD1a^+$DCs expressing HLA-DR, however after radiotherapy, HLA-DR surface expression significantly decreased in all three patients (Figure 7.4e and 7.4f), see Table 7.1.

Table 7.2: Statistical significance and direction of change of data from Figures 7.3 and 7.4

Population	Direction of Change	P Value
$CD14^-$ DCs		
$\%CD11c^+$	↓↓↓	**p = 0.02**
CD11c MFI	↓↓↓	**p = 0.005**
$\%CD40^+$	↑↑↓	p = 0.40
CD40 MFI	↑↑↑	p = 0.43
$\%CD83^+$	↓↓↓	**p = 0.04**
CD83 MFI	↓↓↓	**p = 0.04**
$\%CD86^+$	↓↓↓	**p = 0.03**
CD86 MFI	↓↓↓	p = 0.27
$CD11c^+$ DCs		
$\%CD1a^+$	↑↑↓	p = 0.66
CD1a MFI	↑↑↓	p = 0.36
$CD11c^+CD1a^+$DCs		
$\%CD80^+$	↑↑↓	p = 0.48
CD80 MFI	↑↔↔	p = 0.30
$\%HLA\text{-}DR^+$	↔↔↔	p = 0.50
HLA-DR MFI	↓↓↓	**p = 0.002**

7.2.4 Radiotherapy interferes with the ability of DCs to up-regulate CD11c, CD83, CD86 and HLA-DR after stimulation

To determine if MoDCs respond appropriately to stimuli post radiotherapy, MoDCs from the three patients were stimulated with LPS, IFNγ, LPS + IFNγ or CD40L for 24 hours and DCs analysed by flow cytometry for expression of CD1a, CD11c, CD40, CD80, CD83, CD86 and HLA-DR as previously described. Pre-radiotherapy responses were compared to post-radiotherapy responses.

Overall, the data shows that regardless of the stimuli used radiotherapy reduced the percentage of cells positive for CD11c and CD86 (Figures 7.5a and 7.5g); expression levels of these cell surface markers were also reduced (Figure 7.5b, 7.5f and 7.5h), see Table 7.3. Variable results were seen for CD40, with CD40 expression reduced post-radiotherapy in two of the three patients and increased in the third patient (Figures 7.5c and 7.5d).

Table 7.3: Statistical significance of data from Figure 7.5

	LPS	IFNγ	LPS+IFNγ	CD40L
%CD11c$^+$	**p = 0.02**	**p = 0.02**	**p = 0.03**	**p = 0.02**
CD11c MFI	p = 0.076	p = 0.058	p = 0.093	**p = 0.02**
%CD40$^+$	p = 0.26	p = 0.69	p = 0.12	p = 0.39
CD40 MFI	p = 0.59	p = 0.85	p = 0.35	p = 0.44
%CD83$^+$	p = 0.32	**p = 0.02**	p = 0.27	**p = 0.03**
CD83 MFI	p = 0.25	**p = 0.015**	p = 0.29	p = 0.05
%CD86$^+$	**p = 0.002**	**p = 0.006**	**p = 0.004**	p = 0.09
CD86 MFI	**p = 0.015**	p = 0.29	**p = 0.009**	p = 0.058

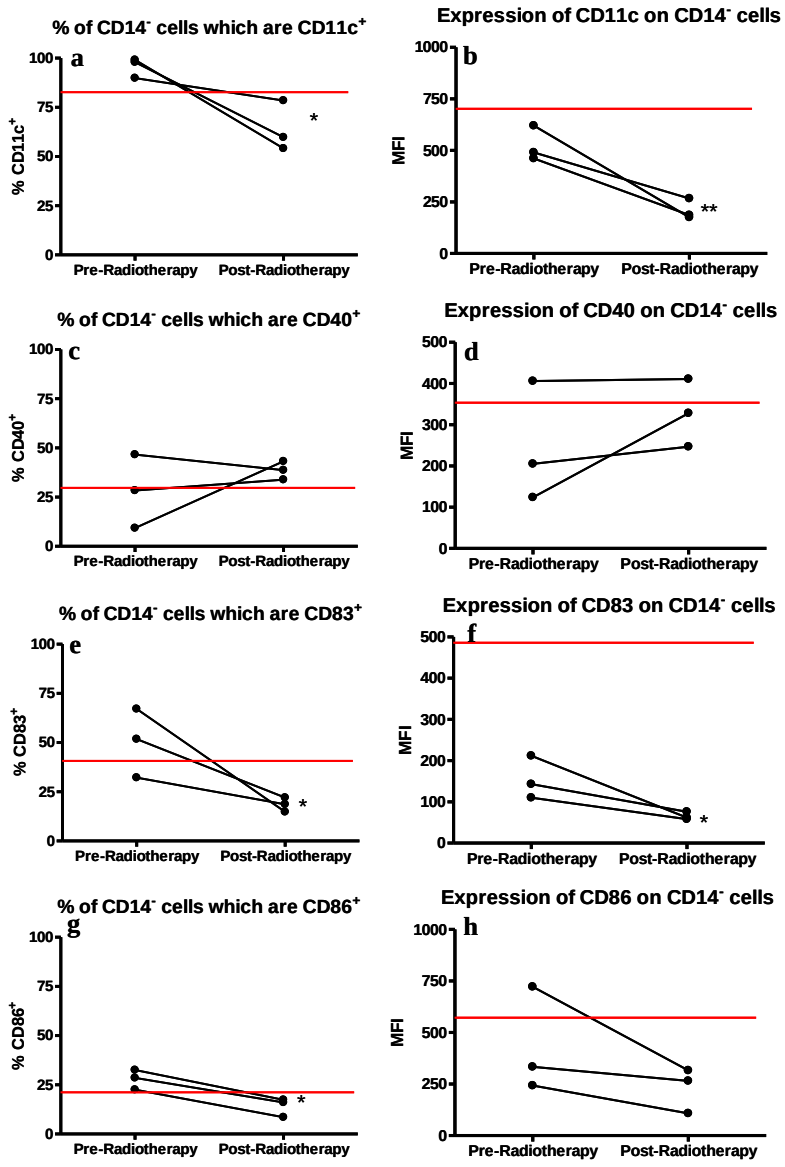

Figure 7.3: CD83 and CD86 expression is reduced on mesothelioma patient iMoDCs following radiotherapy

Human monocytes from mesothelioma patients pre and post radiotherapy were differentiated into iMoDCs using GM-CSF and IL4. Immature MoDCs were collected and cell surface molecules analysed by flow cytometry. $CD14^-$ cells were analysed for the expression of CD11c (a,b), CD40 (c,d), CD83 (e,f) and CD86 (g,h). Pooled data of the percentages of cells positive for CD11c (a), CD40 (c), CD83 (e) and CD86 (g). Surface expression levels were measured and shown as MFIs of CD11c (b), CD40 (e), CD83 (f) and CD86 (h) in mesothelioma patients' (n = 3) MoDCs. Pooled data is shown as mean ± SEM. *$p<0.05$, **$p<0.01$. Average % and MFI of healthy controls is shown as a red line.

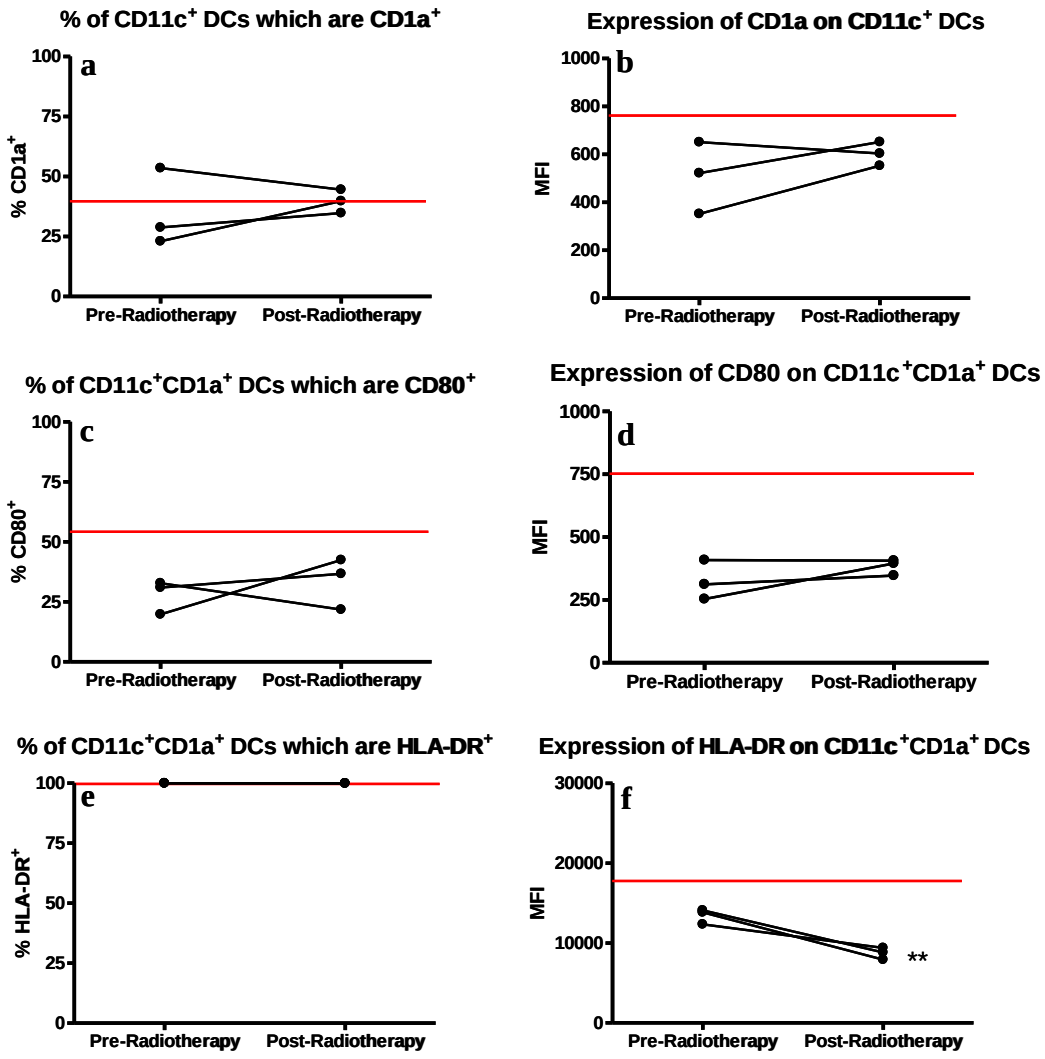

Figure 7.4: Radiotherapy decreases HLA-DR expression on mesothelioma patient iMoDCs.

Immature MoDCs generated from mesothelioma patients, pre and post radiotherapy were analysed by flow cytometry. $CD14^-$ cells were identified and gated for expression of CD11c. $CD11c^+$ DCs were further gated for expression of CD1a (a,b). $CD11c^+CD1a^+$ DCs were analysed for expression of CD80 (c,d) and HLA-DR (e,f). Pooled data of the percentages of cells positive for CD1a (a), CD80 (c) and HLA-DR (e). Surface expression levels were measured and shown as MFIs of CD1a (b), CD80 (e) and HLA-DR (f) in mesothelioma patients' (n = 3) MoDCs. Pooled data is shown as mean ± SEM. **p<0.01. Average % and MFI of healthy controls is shown as a red line.

Regardless of the stimuli used, CD1a expression levels in post-radiotherapy $CD11c^+$ DCs increased, although changes in the percentages of $CD1a^+CD11c^+$ DCs varied (Figures 7.6a and 7.6b). CD80 on post-radiotherapy $CD11c^+CD1a^+$DCs decreased in all three patients in response to LPS +/- IFNγ yet increased or did not change in response to CD40L (Figures 7.6c and 7.6d). Statistically significant decreases were observed in the percentage of post-radiotherapy cells expressing CD80 in response to IFNγ ($p = 0.007$) or LPS + IFNγ ($p = 0.005$; Figure 7.6c). In addition, whilst the percentage of $CD11c^+CD1a^+$ DCS expressing HLA-DR did not change (Figure 7.6e), HLA-DR expression levels significantly and profoundly decreased 2 to 3-fold in response to all stimuli post radiotherapy (Figure 7.6f), see Table 7.4. Whilst the percentage of DCs expressing these markers were similar to their healthy age-matched controls their corresponding expression levels at baseline were below healthy levels and substantially decreased further following radiotherapy.

Table 7.4: Statistical significance of data from Figure 7.6

	LPS	IFNγ	LPS+IFNγ	CD40L
$\%CD1a^+$	p = 0.93	p = 0.57	p = 0.91	p = 0.69
CD1a MFI	p = 0.21	p = 0.11	p = 0.39	p = 0.25
$\%CD80^+$	p = 0.26	p = 0.07	**p = 0.005**	p = 0.67
CD80 MFI	p = 0.42	p = 0.38	p = 0.09	p = 0.26
$\%HLA\text{-}DR^+$	p = 1.0	p = 0.83	p = 1.0	p = 0.21
HLA-DR MFI	**p = 0.32**	**p = 0.32**	**p = 0.32**	**p = 0.32**

7.2.5 Long-term post-surgery recovery of mDC numbers in a mesothelioma patient is lost following radiotherapy

Peripheral blood samples were collected at four time points (Figure 7.7a) from one mesothelioma patient undergoing substantive debulking surgery immediately followed by radiotherapy. Circulating DC populations (pDCs, mDC1s and mDC2s) were enumerated as previously described.

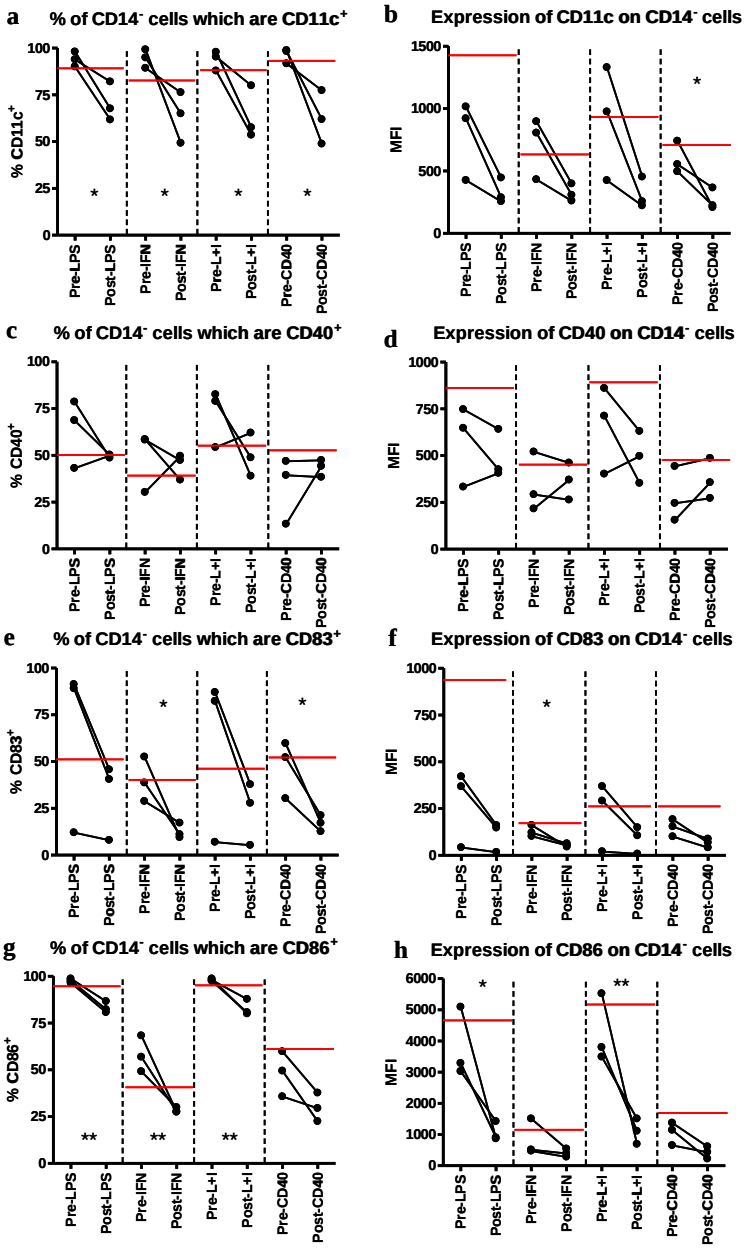

Figure 7.5: Radiotherapy is associated with decreased CD11c, CD83 and CD86 expression on MoDCs in response to stimulation

Immature MoDCs generated from mesothelioma patients pre- and post- radiotherapy were stimulated with LPS and/or IFNγ or CD40L and CD14$^-$ cells analysed by flow cytometry for expression of CD11c (a,b), CD40 (c,d), CD83 (e,f) and CD86 (g,h). Pooled data of the percentages of cells positive for CD11c (a), CD40 (c), CD83 (e) and CD86 (g). Surface expression levels were measured as MFIs of CD11c (b), CD40 (e), CD83 (f) and CD86 (h) in mesothelioma patients (n = 3) MoDCs. Pooled data is shown as mean ± SEM. Average % and MFI of healthy controls is shown as red lines. *p<0.05, **p<0.01

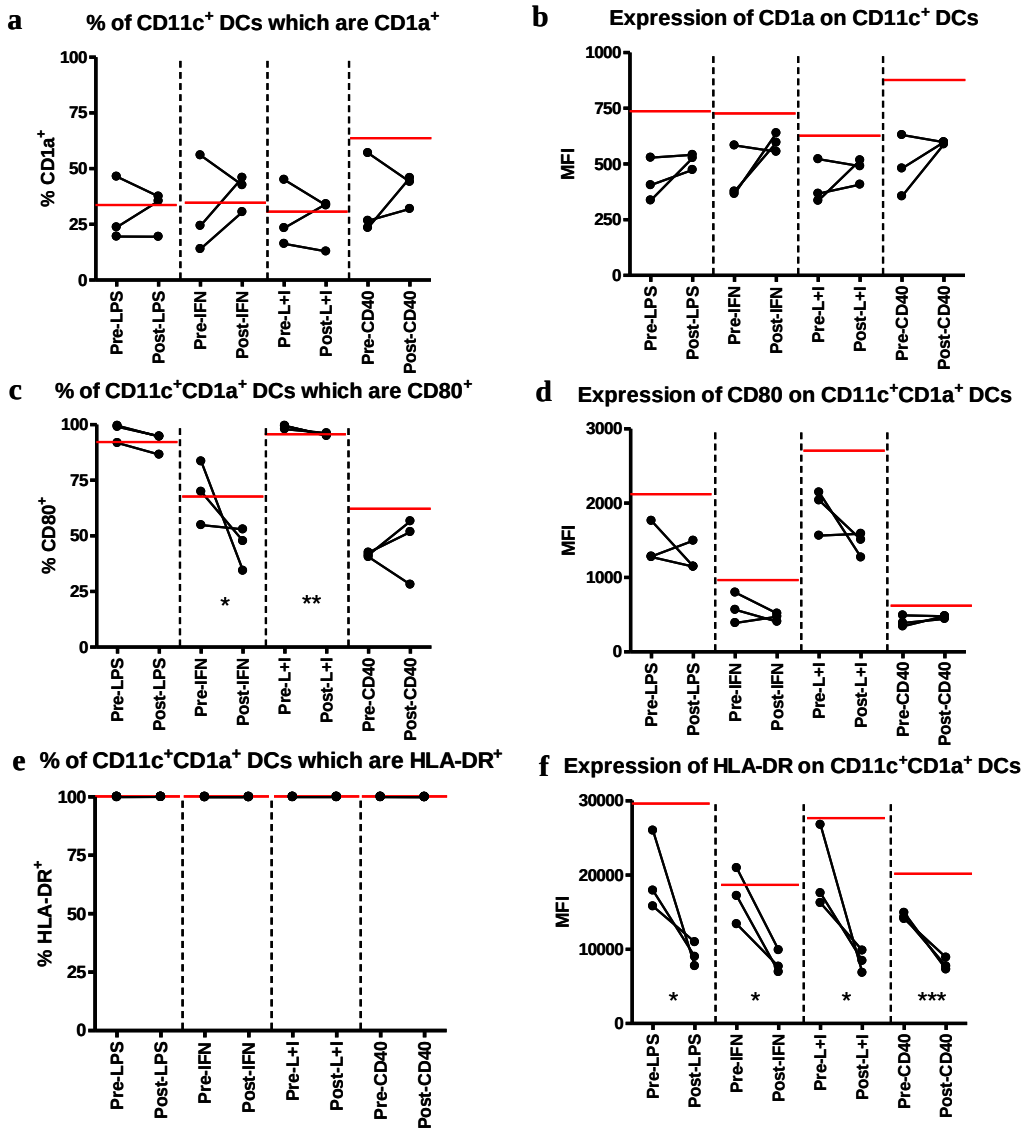

Figure 7.6: Radiotherapy prevents up-regulation of HLA-DR expression on MoDCs following stimulation

Immature MoDCs generated from mesothelioma patients (pre and post radiotherapy) were stimulated with LPS, IFNγ, LPS + IFNγ or CD40L and cell surface molecules were analysed by flow cytometry. $CD14^-$ cells were identified and gated for expression of CD11c. $CD11c^+$ DCs were further analysed and gated on the expression of CD1a (a,b). $CD11c^+CD1a^+$ DCs were analysed for the expression of CD80 (c,d) and HLA-DR (e,f). Pooled data of the percentages of cells positive for CD1a (a) and CD80 (c). Surface expression levels were measured and shown as MFIs of CD1a (b), CD80 (d) and HLA-DR (e) in mesothelioma patients' (n = 3) MoDCs. Pooled data is shown as mean ± SEM. Average % and MFI of healthy controls is shown as red lines. *p<0.05, **p<0.01, ***p<0.001

Pre-surgery numbers of mDC1, mDC2 and pDCs were lower than healthy controls with the most marked pre-operative suppression seen in the mDC2 population (Figures 7.7b-d). Three days following surgery, there was a marked decrease in pDC (Figure 7.7d) and mDC1 (Figure 7.7b) numbers, and no change in mDC2 (Figure 7.7c). By eight weeks post-surgery the number of pDCs was approaching levels seen prior to surgery, but nonetheless did not reach the mean level seen in healthy controls. The numbers of mDC1s increased above baseline to levels seen in healthy controls. Interestingly, the mDC2 population increased almost 3 fold higher than that seen prior to surgery, also to the same level seen in healthy controls. Radiotherapy started 10 weeks post-operatively and consisted of 60 Gy in 30 fractions. Eight weeks following the beginning of radiotherapy all three populations had decreased to levels lower than that observed post surgery. There was no tumour progression during this period. This data implies that, for at least this patient, surgery decreased factors that were either inhibiting the production of mDCs or inducing apoptosis in mDCs.

7.2.6 Neither surgery or radiotherapy restore appropriate antigen processing responses in mesothelioma patient MoDCs

The data in Figure 7.2c shows a decrease in MoDC antigen processing ability in response to stimuli after radiotherapy, implying restoration of the DC maturation process. To investigate whether surgery prior to radiotherapy improves DC maturation responses, the DQ assay was performed on immature and stimulated MoDCs (LPS, IFNγ, LPS + IFNγ or CD40L) generated from blood collected at the four time points shown in Figure 7.7a.

Examination of immature DCs (whose primary function is to take up and process antigen) shows that neither surgery nor radiotherapy interfered with the ability of iMoDCs to process antigen in this individual (Figures 7.8a and 7.8b). In healthy controls, DC activation is associated with a dramatic reduction in antigen processing. This normal response was seen transiently following surgery with LPS alone, and was sustained post-radiotherapy. Immediately following both surgery and radiotherapy, the anticipated reduction in antigen processing by stimulation with CD40L or IFNγ

appeared muted in comparison to pre-surgery (Figures 7.8a and 7.8b). Although a slight recovery was observed for CD40L (but not IFNγ) stimulation of MoDCs generated 8 weeks after surgery. The data suggests that in this individual there was a transient impact directly following surgery and radiotherapy on the antigen processing capacity of MoDCs stimulated with either CD40L or IFNγ.

7.2.7 Radiotherapy reverses CD1a, CD11c, CD80 and CD83 responses to stimuli following surgery in a patient undergoing multiple therapies

To investigate whether the increased DC numbers following surgery translated to an improvement in function, MoDCs generated at each time point were stimulated with LPS, IFNγ, LPS + IFNγ or CD40L and CD1a, CD11c, CD40, CD80, CD83, CD86 and HLA-DR expression determined by flow cytometry.

Overall, the data shows that regardless of the stimuli used surgery generally improved phenotypic responses whilst radiotherapy reduced or even ablated these responses in this patient. For example, CD11c increased at the early and later time points following surgery yet decreased after radiotherapy (Figures 7.9a and 7.9b). Similarly, the percentage of iMoDCs expressing CD40 (Figure 7.9c) increased following surgery and decreased after radiotherapy; although CD40 expression levels remained high after both treatment modalities (Figures 7.9d and 7.9e). The effect was even more pronounced for CD83 which increased in response to some stimuli post surgery; this response was profoundly ablated after radiotherapy (Figures 7.9e and 7.9f). Whilst the percentage of $CD86^+$ DCs appeared more robust following surgery and radiotherapy, CD86 expression levels reduced substantially after radiotherapy (Figures 7.9g and 7.9h). The data show that activation by LPS +/- IFNγ induced the strongest phenotypic responses followed by CD40L, then IFNγ alone.

The percentage of MoDCs expressing CD1a increased post-surgery but decreased following radiotherapy (irrespective of stimulation), yet CD1a expression levels

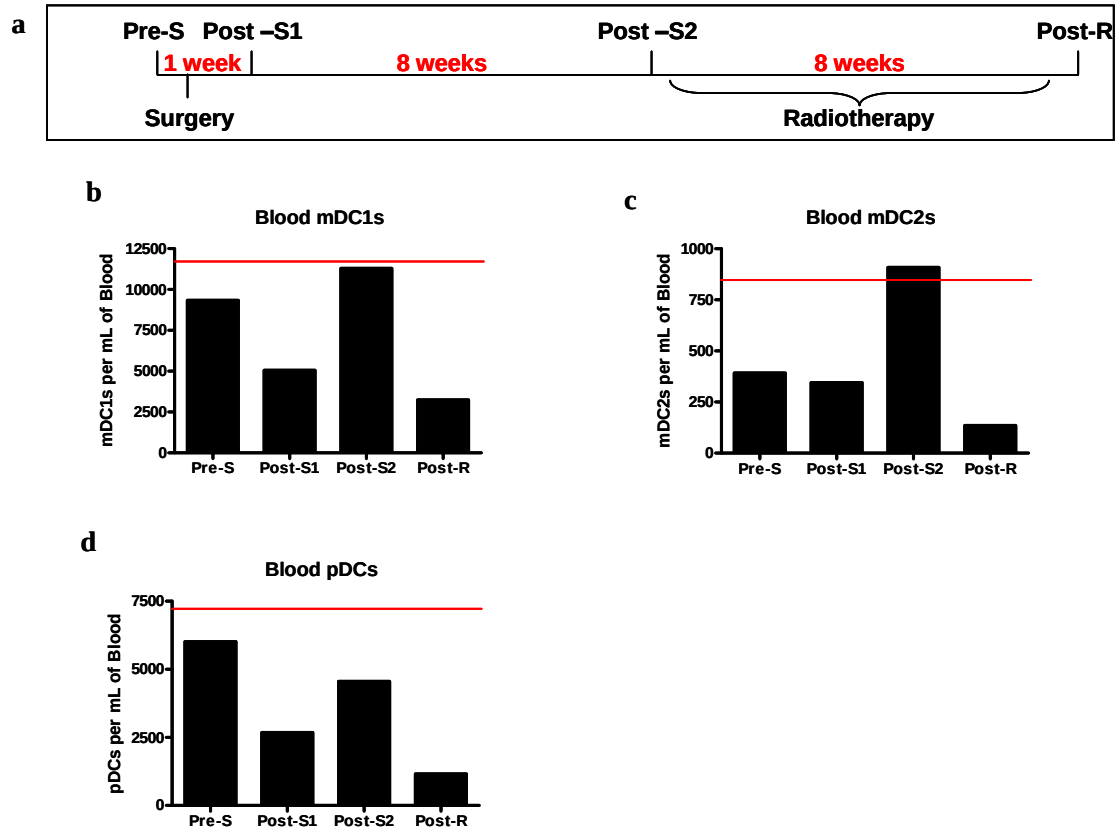

Figure 7.7: Long-term post-surgery recovery of mDC numbers in a mesothelioma patient is lost following radiotherapy

Blood samples were collected at four time-points from a patient undergoing debulking surgery and follow-up radiotherapy (a). Whole blood was stained for blood DC subpopulations and analysed by flow cytometry. Blood DC subpopulations were identified by high expression of BDCA-1 (b: mDC1), BDCA-3 (c: mDC2) and BDCA2 (d: pDC). Absolute counts were determined by multiplying the number of DCs by the number of PBMCs determined by counting on a haemocytometer. The absolute number of circulating, mDC1, mDC2 and pDCs measured as the number of DCs per ml of blood were plotted. The red line represents DC levels in aged-matched healthy controls.

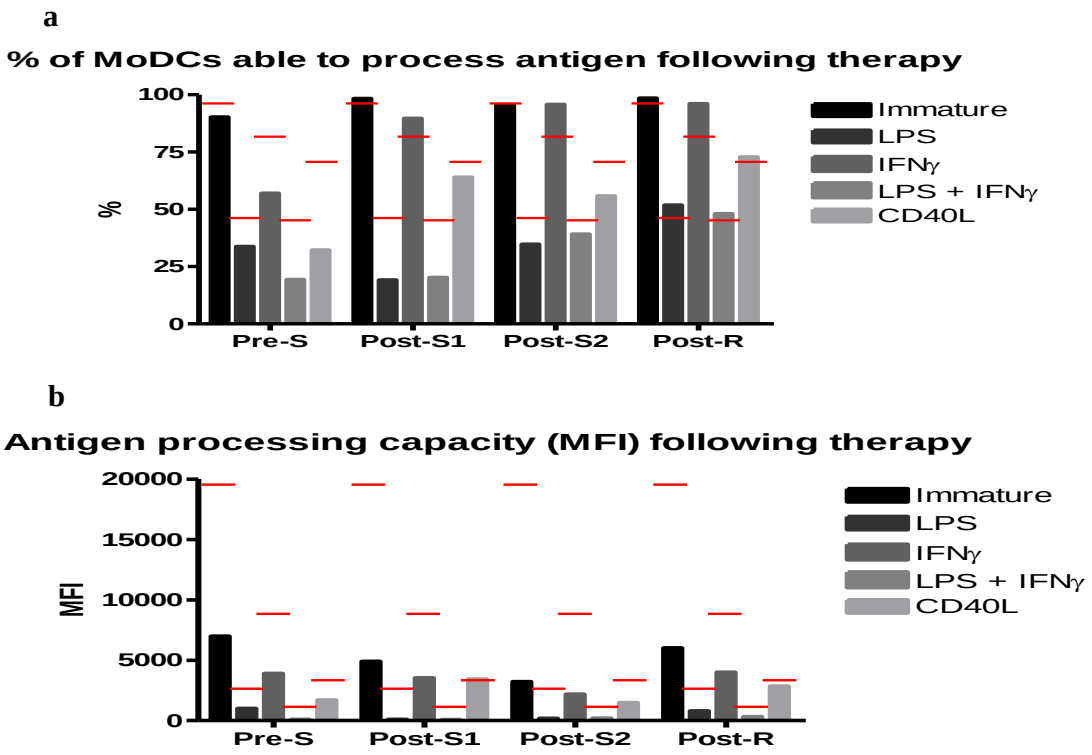

Figure 7.8: Neither surgery or radiotherapy restores the antigen processing ability of mesothelioma patient MoDCs

MoDCs were generated from blood monocytes isolated from a mesothelioma patient (n = 1) undergoing surgery and radiotherapy. Unstimulated and stimulated MoDCs were then incubated for 1 hour with FITC-labeled DQ-Ova. The capacity to process antigen was measured by flow cytometry. Data (a) showing the % of DCs able to process antigen and the relative antigen processing capacity (MFI) and (b) average % and MFI of healthy controls is shown as red lines.

remained relatively stable (Figures 7.10a and 7.10b). Whilst the percentage of $CD11c^{+}CD1a^{+}$ MoDCs expressing CD80 and the expression levels of CD80 increased slightly following surgery, any increase was lost following radiotherapy (Figures 7.10c and 7.10d). In addition, the percentage of $CD11c^{+}CD1a^{+}$ DCs expressing HLA-DR remained stable at 100% of cells expressing HLA-DR before or after therapy, HLA-DR expression levels decreased immediately post-surgery, and were showed no sign of recovery post-radiotherapy (Figure 7.10e and 7.10f). The data shows that, at least in this individual, surgery, if anything, only offered a transient improvement in the phenotype of iMoDCs and their response to activation, which was lost following radiotherapy.

7.2.8 Mesothelioma patient pleural fluid has decreased mDC1s and increased pDCs compared to blood

As shown in chapter 6, tumour-derived factors interfere with DC phenotype and function. DCs and their precursors in pleural fluid might be closer to the tumour microenvironment and therefore exposed to higher concentrations of these factors than bone marrow and blood DC precursors. Thus, pleural fluid DCs might be more dysfunctional than their blood counterparts.

Pleural fluid was obtained from two patients who had indwelling pleural catheters during the course of the study for standard clinical indications. DCs enumerated from the pleural fluid of these two patients were compared to the number of circulating DCs enumerated from a concurrent blood draw. In addition, pleural fluid from a third patient without a concurrent blood draw was included. No trends were seen in the two patients, with the proportion of pleural fluid DC subsets not corresponding closely to the proportion of blood DCs of the same subset at the same timepoint (Figures 7.11a, 7.11c and 7.11e). Whilst the mean proportion of mDC2s and pDCs was decreased in patient blood as compared with healthy controls, this did not correspond with a low number in the pleural fluid (Figures 7.11b and 7.11c). Indeed, two of the patients had either equal or higher numbers of mDC2s and pDCs in their pleural fluid than that observed in healthy control blood. Interestingly the proportion of mDC1 in pleural fluid was lower in

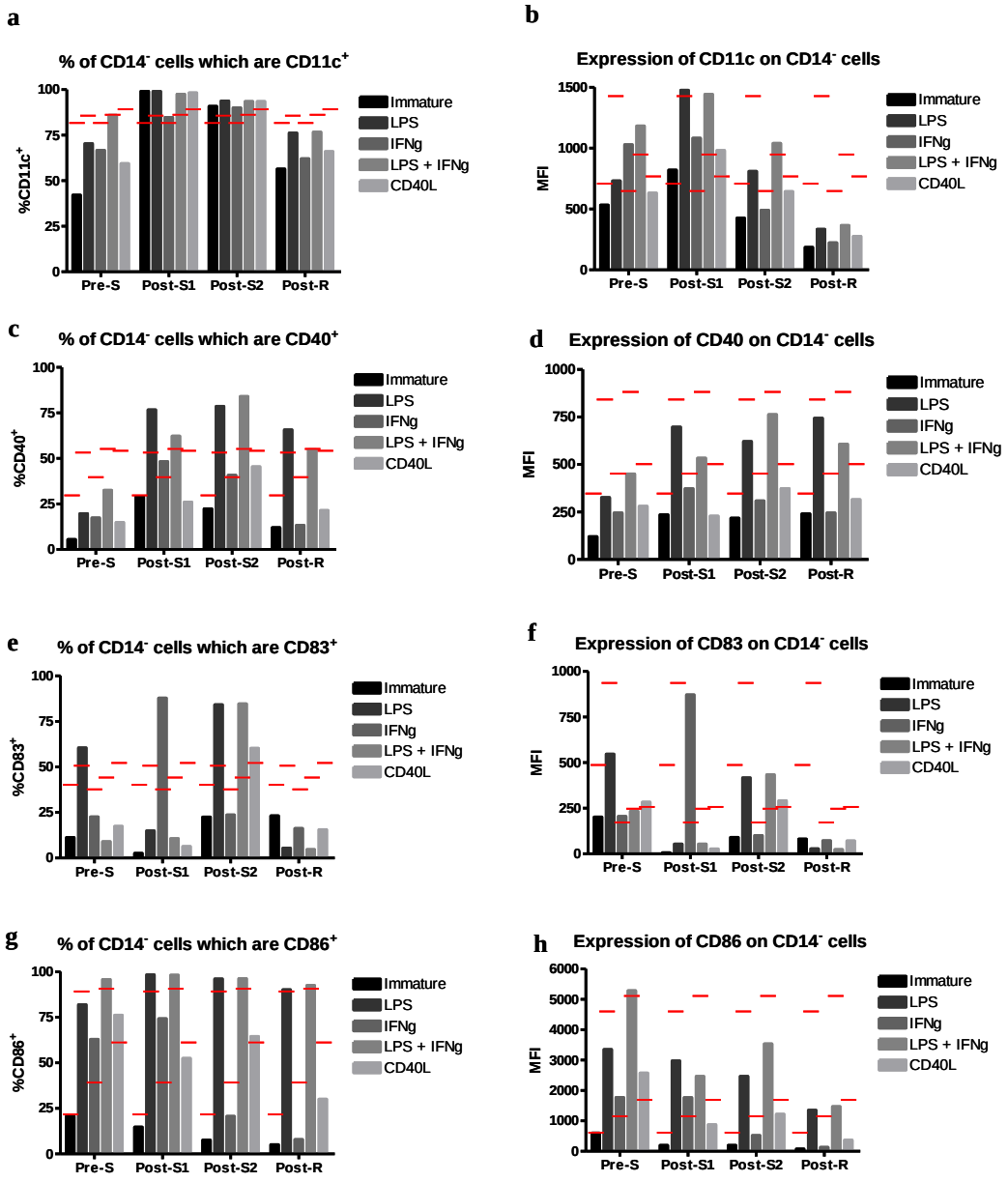

Figure 7.9: CD11c and CD83 responses to stimuli following surgery are lost post radiotherapy

Human monocytes isolated from whole blood collected from a mesothelioma patient undergoing therapy (Figure 7.7a), were differentiated into iMoDCs. Immature MoDCs were stimulated with LPS, IFNγ, LPS + IFNγ or CD40L and cell surface molecules were analysed by flow cytometry. Plots are data showing the percentage of $CD14^-$ cells expressing CD11b (a), CD40 (c), CD83 (e) and CD86 (g). Surface expression levels were measured and shown as MFIs of CD11c (b), CD40 (d), CD83 (f) and CD86 (h). Average % and MFI of healthy controls is shown as red lines.

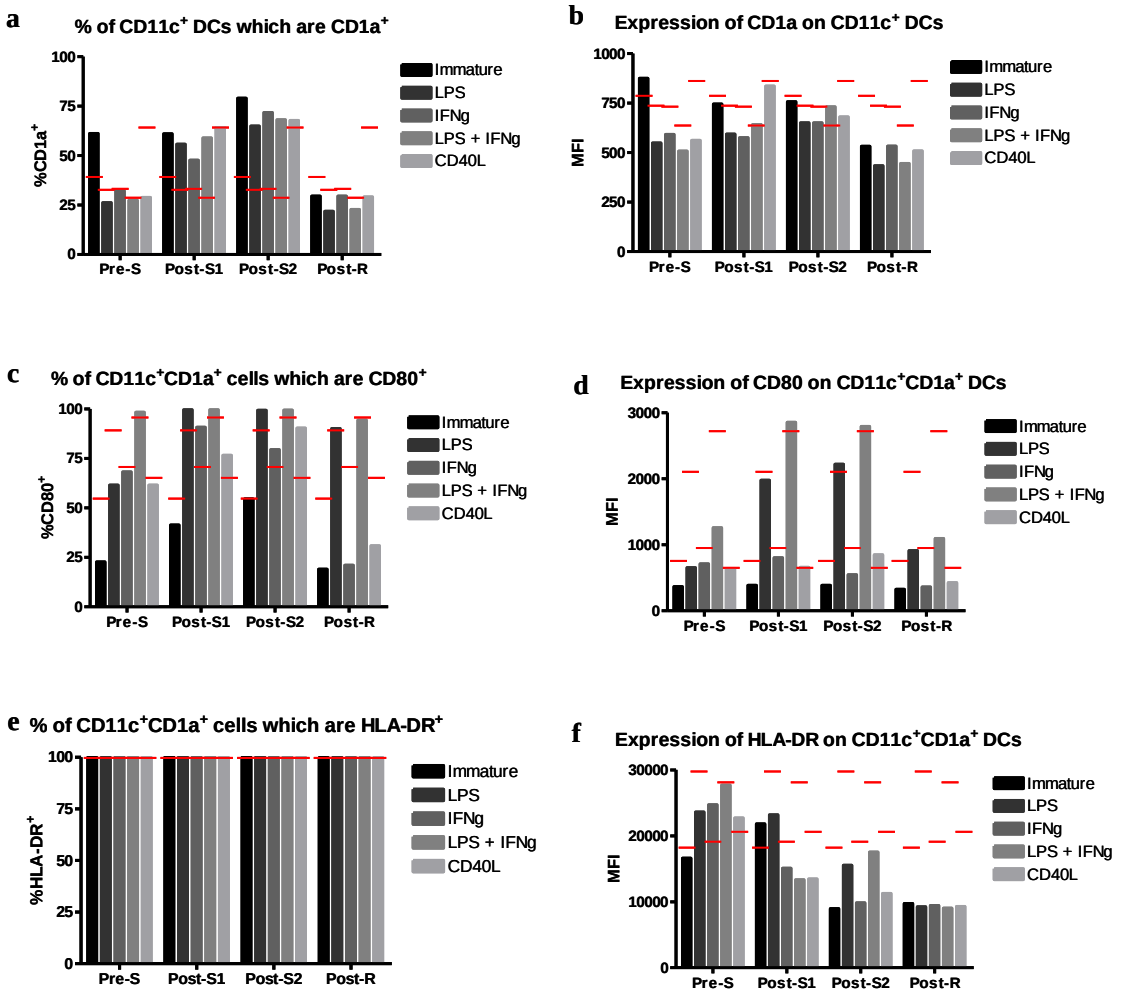

Figure 7.10: The increased CD1a and CD80 expression observed following surgery is absent post radiotherapy

Immature MoDCs from a mesothelioma patient undergoing therapy (Figure 7.7a) were stimulated with LPS, IFNγ, LPS + IFNγ or CD40L and cell surface molecules were analysed by flow cytometry. $CD11c^+$ DCs were analysed for both the percentage of cells expressing CD1a (a) and the surface expression levels (MFI) of CD1a (b). $CD11c^+CD1a^+$ DCs were further analysed for the percentage of cells expressing CD80 (c) and HLA-DR (data not shown) and the surface expression levels of CD80 (d) and HLA-DR (e,f). Average % and MFI of healthy controls is shown as red lines.

all three patients than the proportions expected in healthy control blood, corresponding to the relatively lower proportions of mDC1 seen in patient blood (Figure 7.11a).

7.2.9 MoDCs from pleural fluid showed a decreased capacity to process antigen relative to MoDCs from blood.

DC maturation (i.e. higher expression levels of co-stimulatory and maturation markers) should be associated with a decrease in antigen processing. Immature MoDCs were generated from concurrently obtained blood and pleural fluid from a single patient. Almost 100% of blood and pleural fluid iMoDCs were able to process antigen, however the levels at which they were processing antigen (MFI) were markedly reduced in the pleural fluid iMoDCs (Figures 7.12a and 7.12b). Following stimulation, blood-derived MoDCs appeared to respond better to LPS or the LPS/IFNγ combination, demonstrating a greater decrease in antigen processing (Figures 7.12a and 7.12b), implying maturation. Only IFNγ alone induced a greater reduction in antigen processing in pleural MoDCs. Interestingly, following stimulation with CD40L, MoDCs from pleural fluid and blood showed no difference in their magnitude of response with an overall weaker response implying partial maturation.

7.2.10 Mesothelioma patient pleural fluid DCs are more activated than blood DCs

Monocytes were collected from circulating blood and pleural fluid from the same patient. MoDCs were then generated from the two populations of monocytes (circulating and pleural resident) and analysed for surface molecule expression as previously described.

More immature $CD14^-$ DCs in pleural fluid relative to blood expressed CD11c , CD83 , and CD86 (Figure 7.13a, 7.13e and 7.13g); this was associated with apparent higher expression levels of CD11c , CD40 , CD83 and CD86 (Figures 7.13b, 7.13d, 7.13f and 7.13h) in DCs derived from pleural fluid. No differences were seen between immature $CD14^-$ cells from pleural fluid versus blood monocytes expressing CD40, yet increased CD40 was seen on pleural DCs (Figures 7.13c and 7.13d).

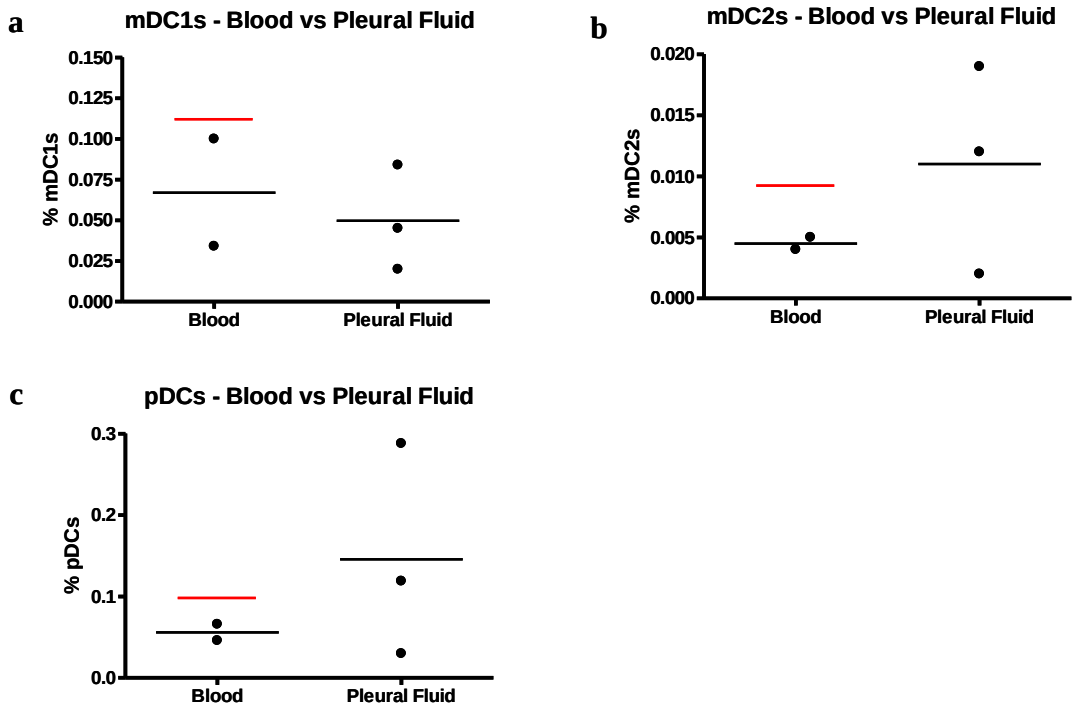

Figure 7.11: Mesothelioma patient pleural fluid has a decreased percentage of mDC1s, and an increased percentage of pDCs compared to patient blood

Whole blood (n = 48) and pleural fluid (n = 3) were stained for blood DC subpopulations and analysed by flow cytometry. Blood DC subpopulations were identified by high expression of BDCA-1 (a: mDC1), BDCA-3 (b: mDC2) and BDCA2 (c: pDC). The percentage of each DC population was determined as the number of DCs divided by the total number of leukocytes (represented as a percentage). Each dot represents an individual's blood or pleural fluid. Average % of DCs in all 48 mesothelioma patients' blood is shown as a red line.

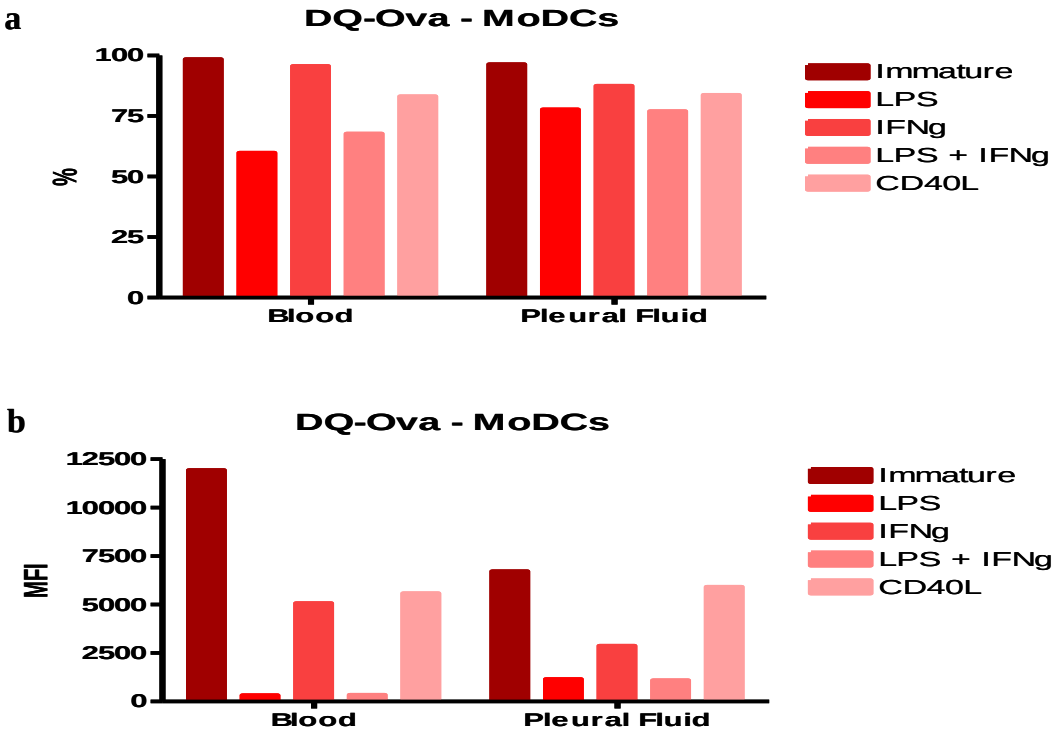

Figure 7.12: MoDCs generated from pleural fluid have a decreased capacity to process antigen in comparison to MoDCs from the same patient's blood

MoDCs were generated from both blood monocytes and pleural effusion monocytes isolated from a mesothelioma patient (n = 1). Unstimulated and stimulated MoDCs were then incubated for 1 hour with FITC-labeled DQ-Ova. The capacity to process antigen was measured by flow cytometry. Data (a) showing the % of DCs able to process antigen and the relative antigen processing capacity (MFI) (b).

The proportion of immature $CD11c^+$ DCs expressing CD1a did not differ between blood and pleural fluid. Likewise, the percentage of immature $CD11c^+CD1a^+$ DCs expressing HLA-DR was similar. The percentage of $CD11c^+CD1a^+$ DCs expressing CD80 was higher for pleural fluid DCs. The same immature pleural DCs had higher expression levels for CD1a, CD80 and HLA-DR (Figures 7.14a-f).

Following stimulation with LPS, IFNγ, LPS + IFNγ or CD40L, the percentage of $CD14^-$ pleural fluid MoDCs expressing CD40 was similar to blood derived $CD14^-$ MoDCs (Figure 7.13c). A greater percentage of pleural fluid $CD14^-$ MoDCs expressed CD11c, CD83 and CD86 (Figure 7.13a, 7.13e and 7.13g). In addition, surface expression (irrespective of stimulus used) of CD11c, CD40 and CD83 was higher on pleural fluid derived $CD14^-$ MoDCs (Figures 7.13b, 7.13d and 7.13f). Interestingly, expression of CD86 post stimulation was only higher on pleural fluid derived $CD14^-$ MoDCs stimulated with IFNγ alone or CD40L (Figure 7.13h). There were no differences in the percentage of $CD11c^+$ DCs expressing CD1a, or $CD11c^+CD1a^+$ DCs expressing HLA-DR (Figures 7.14a and 7.14e). Expression of CD1a on $CD11c^+$ DCs was higher for pleural fluid MoDCs, and expression of CD80 and HLA-DR on pleural fluid $CD11c^+CD1a^+$ DCs was also greater (Figures 7.14b, 7.14d and 7.14f). These data imply that MoDCs generated from the pleural fluid monocytes from this patient were more activated than those generated from blood.

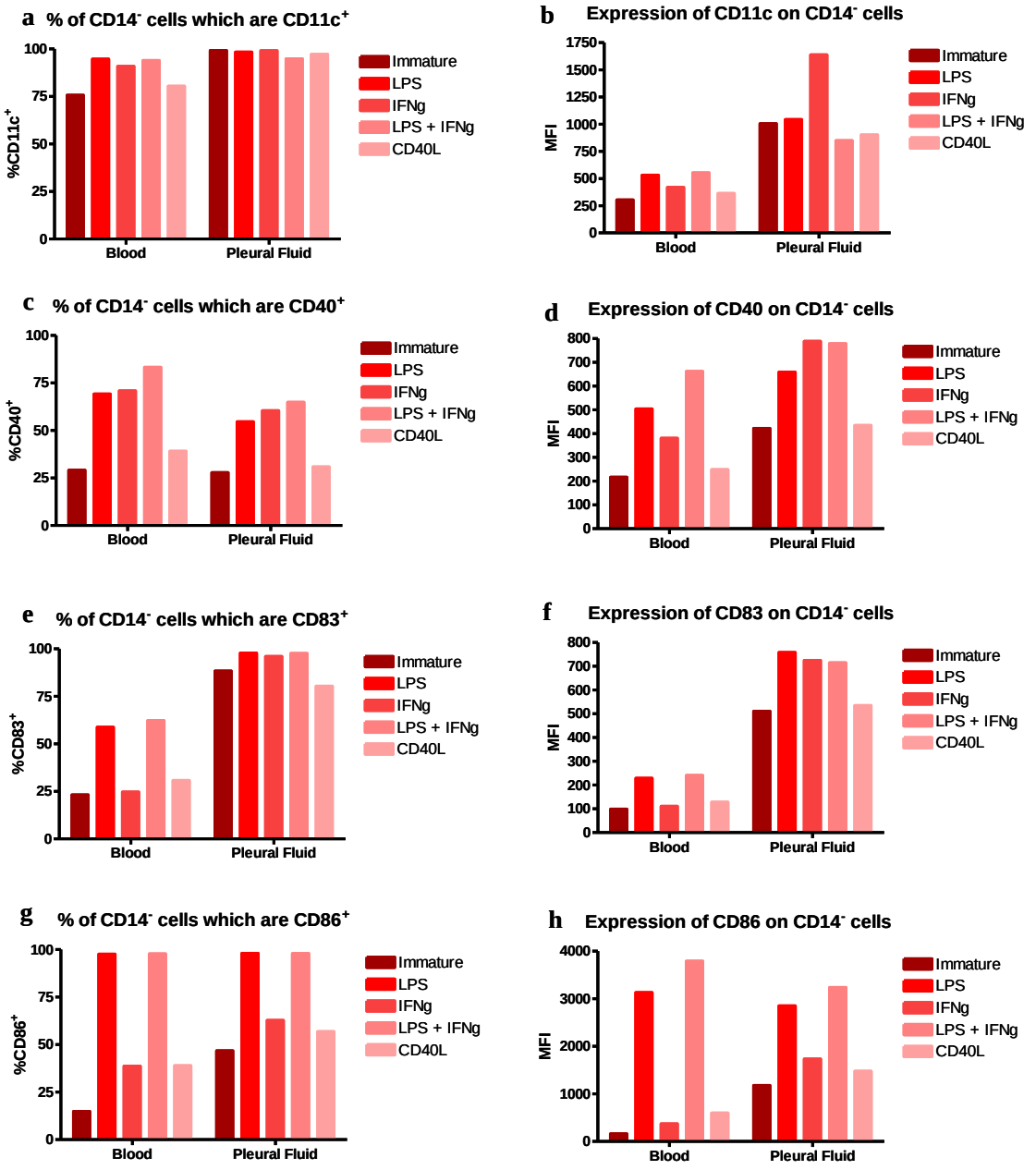

Figure 7.13: Mesothelioma patient pleural fluid DCs are more activated than peripheral blood DCs

Immature MoDCs generated from a mesothelioma patient's blood and pleural fluid monocytes were stimulated with LPS, IFNγ, LPS + IFNγ or CD40L and cell surface molecules were examined by flow cytometry. $CD14^-$ cells were analysed for the percentage of cells expressing CD11c (a), CD40 (c), CD83 (e) and CD86 (g) and the surface expression levels (MFI) of CD11c (b), CD40 (d), CD83 (f) and CD86 (h).

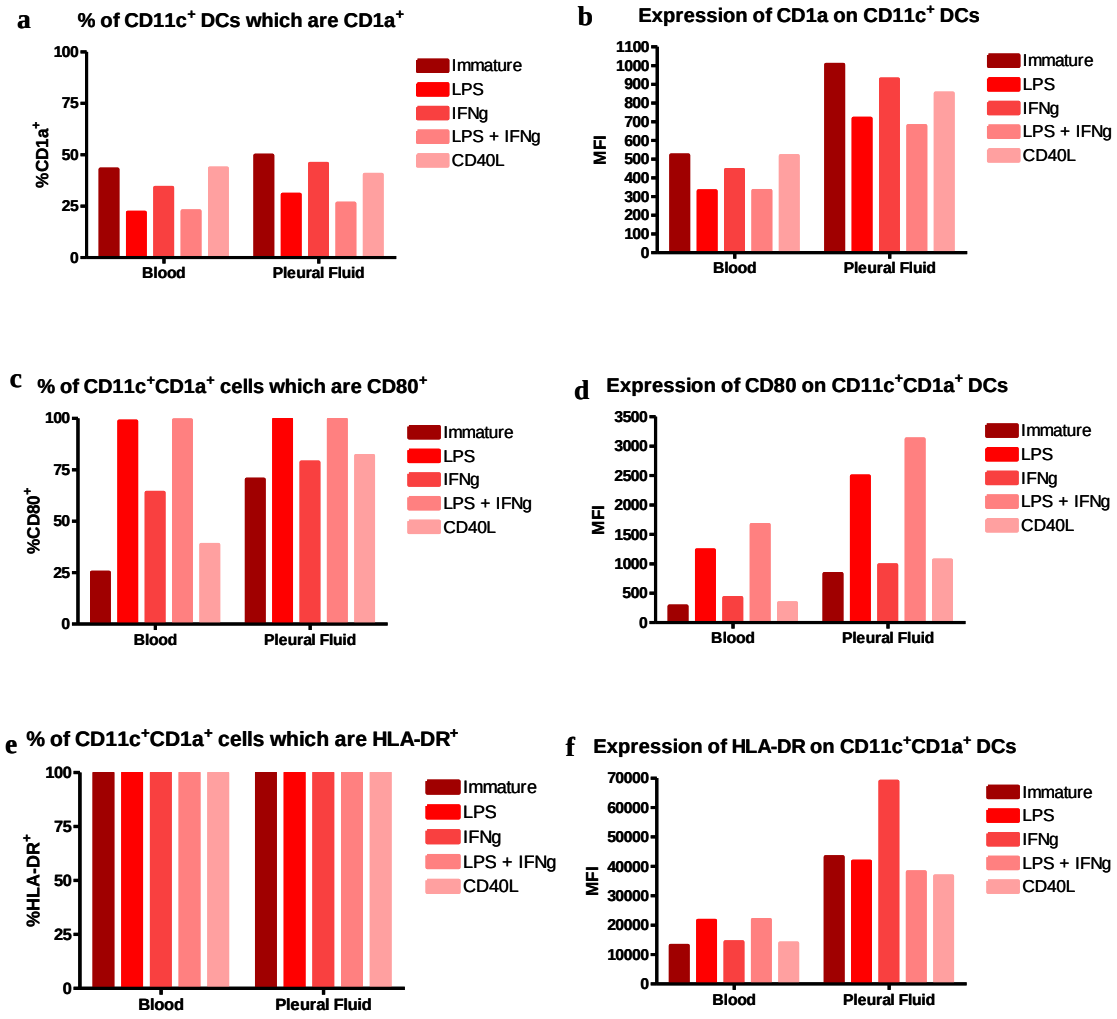

Figure 7.14: Mesothelioma patient pleural fluid DCs have higher expression of CD1a, CD80 and HLA-DR in comparison to blood DCs

MoDCs generated from monocytes from either blood or pleural effusion of a mesothelioma patient (n = 1) were stimulated with LPS, IFNγ, LPS + IFNγ or CD40L and cell surface molecules were examined by flow cytometry. $CD11c^+$ DCs were analysed for the expression of CD1a (a,b). $CD11c^+CD1a^+$ DCs were then further analysed for the expression of CD80 (c,d) and HLA-DR (e). The data shows the percentages of cells expressing CD1a (a) and CD80 (c) as well as surface expression levels (MFIs) of CD1a (b), CD80 (d) and HLA-DR (f).

7.3 Discussion

Current therapies for mesothelioma can include surgery and radiotherapy. Radiotherapy is often administered following surgery, but more frequently is administered alone for palliative reasons. Both therapies reduce tumour burden and therefore have the potential to alleviate the suppressive effects of mesothelioma-derived factors on DCs. To date no study has investigated the effect of surgery and radiotherapy on DCs in mesothelioma patients.

The results in the previous chapters showed that mesothelioma patients have significantly decreased numbers of circulating DCs, suggesting difficulties in mounting an immune response. The data in this chapter with patients undergoing only radiotherapy for palliative reasons showed an elevation in the number of circulating DCs in 2 out of 3 patients; however, their levels were never restored to that of healthy age-matched controls. As shown in chapter 3, iMoDCs generated from mesothelioma patients have a clear defect in their antigen processing capacity; this was further decreased following radiotherapy in the three patients investigated in this chapter. Post-radiotherapy activated MoDCs appeared to be maturing appropriately by further decreasing antigen processing capacity. However, this response may be of little use if the DC could not first process antigen. Whilst there are no studies showing the effect of patients undergoing tumour targeted radiotherapy on DC antigen processing, several studies have investigated the effect of ionizing radiation on DC antigen processing in vitro. Indeed, similar to the results in this chapter, Liao et al. (2004) described a decrease in antigen processing and presentation following exposure of murine bone marrow derived DCs to high-dose ionizing radiation, which was likely caused by inhibition of proteasome activity. It is unclear whether radiotherapy could induce a similar inhibition of proteasome activity, as it is also possible that premature DC maturation is induced due to the induction of danger molecules associated with tissue damage.

Ionizing radiation has been shown to modulate the activation status of DCs and in vivo studies administering high-dose radiotherapy directly to tumours found that a release of tumour antigens (such as HMGB1) activated peripheral DCs through TLRs (Apetoh et

al., 2007). The data from our small number of patients shows decreases in CD11c, CD83, CD86 and HLA-DR, implying that radiotherapy did not induce DC maturation. This is in agreement with Reuben et al (2004) and Cao et al (2004), where direct in-vitro exposure of high-dose ionizing radiation led to a decrease in CD80 and CD86 expression on human MoDCs and no up-regulation of CD11c, CD83, CD86 and HLA-DR. Whilst only a limited number of patients were involved in this study, the data reveals novel findings suggesting that radiotherapy impairs the phenotype and function of mesothelioma patient MoDCs. Further studies in larger patient numbers are required to confirm this observation.

The next part of this study asked whether patients who were treated surgically prior to radiotherapy experienced beneficial immune effects in terms of DC function. Studies investigating the effect of debulking surgery for cancer patients on MoDCs have described varying results. Pinzon-Charry (2007) reported decreases in the number of HLA-DR$^+$ DCs immediately post surgery, whilst Ma et al (2009) observed a decrease in the number of mDCs, whilst pDCs were unaffected by surgery. The case study addressed in this chapter showed a decrease in both mDC and pDC numbers post surgery, with a rebound in mDC numbers to higher than pre-surgery levels eight weeks post-surgery. The change observed in mDCs is in agreement with Ma et al (2009) who also measured the highest numbers of mDCs eight weeks after surgery. In contrast, whilst Ma et al did not see changes in pDCs numbers, this case study observed a decrease in pDCs immediately after surgery with a partial recovery eight weeks later. Irrespective of the numbers of DCs reached eight weeks post-surgery, follow-up radiotherapy led to a major loss of all DCs to below pre-surgery levels in this patient. Similarly, the study by Pinzon-Charry also observed a decrease in HLA-DR$^+$ DCs after post-surgery and radiotherapy. However, Ma et al showed increases in mDCs post surgery and radiotherapy, with no change in pDCs.

Post-surgery iMoDCs expressed a less mature phenotype, indicated by reduced CD83 and CD86. Others have reported similar data in other cancers (Brusa et al., 2011, Della Bella et al., 2003, Feng et al., 2012). This may reflect reduced levels of tumour-

associated inflammatory molecules such as IL-6. Nonetheless, surgery did not restore antigen-processing function by mesothelioma-derived iMoDCs. Indeed, antigen processing dropped slightly after surgery before returning to pre-surgery levels post-radiotherapy. Irrespective of the contradictory results, data from this single patient suggests the potential for a window between debulking surgery and radiotherapy where recovery of circulating DCs offers an opportunity for immunotherapy. Further studies are required to systematically investigate this with appropriate controls.

The final part of this chapter was a preliminary study of DCs in pleural fluid. The results from chapter 6 showed that exposure to mesothelioma tumour-derived factors induced significant defects in healthy MoDCs. Therefore proximity to the tumour site could induce a higher degree of impairment. Other studies have shown modulation of DCs due to proximity to the tumour. Whilst these studies observed increases in the number of mDCs in the tumours, these mDCs were observed to have reduced expression of co-stimulatory molecules and DC-LAMP (a protein that assists in MHC class II processing) (Bergeron et al., 2006, Gigante et al., 2009, Nestle et al., 1997). Chapter 4 showed significant decreases in the number of circulating DC subsets in mesothelioma patients. This decrease could be due to: (a) an increase in DC apoptosis as seen in breast cancer patients (Pinzon-Charry et al., 2006) (b) a decrease in the generation of DCs (Shurin et al., 2001a); or (c) recruitment and entrapment of DCs at the tumour site (Gigante et al., 2009). This study showed that pleural fluid surrounding the tumour resulted in increased number of pDCs in association with a decrease in mDC1s.

To date, this is the first study to investigate whether mesothelioma tumour proximity affects the antigen processing ability of DCs. The data shows a further decrease in antigen processing ability for iMoDCs generated from pleural fluid monocytes in comparison to blood MoDCs. In addition, whilst responses to IFNγ appeared improved, responses to LPS and CD40L were muted. The data suggests that pleural fluid MoDCs are more mature than their blood counterparts, which is supported by the more mature phenotype of these MoDCs. Interestingly blood DCs from this patient responded strongly to CD40L, whilst those obtained from pleural fluid did not. This could indicate

an earlier stage of disease, as the blood MoDCs investigated in chapter 6 were very poor responders to CD40L.

In conclusion, these results indicate that the systemic effect observed by mesothelioma on patient DCs (in regards to numbers and function) appears to be more concentrated in closer proximity to the tumour microenvironment. This results correlates well with the data presented in the previous chapter, showing impairments to DCs due to direct exposure to tumour supernatant. Furthermore, whilst both surgery and radiotherapy offer palliative treatments for the patients, there are some indications that they may transiently negatively modulate DC numbers and function, although limited recovery was observed eight weeks post surgery. As these results are limited in the number of patients involved and lack test-retest controls over a similar time period; further studies are required investigating both an increased number of patients and a longer window of study.

8 FINAL DISCUSSION

8.1 Discussion

This thesis investigated how aging might impact on the immune system in people with mesothelioma, a cancer that is predominantly seen in the elderly. Changes to circulating DC subsets and DCs derived from precursor monocyte cells were examined. The data show that pDCs numbers are significantly reduced in the age group in which mesothelioma starts to emerge. However, in people with mesothelioma mDC1, mDC2 and pDC numbers are further compromised. To uncouple the effect of mesothelioma from age-related effects, in vitro studies examining the effect of mesothelioma tumour-cell-derived factors on MoDCs from a young (30 year old) healthy donor were examined. Furthermore, CD40L was investigated for its potential to rescue DCs impaired by age and/or mesothelioma. Finally, surgical and radiotherapy case studies were examined to look for opportunities to include DC-targeting immunotherapies as part of the treatment regimen for mesothelioma.

The data show that whilst the aging process contributes to reducing numbers of circulating pDCs, mesothelioma amplifies this effect and also reduces the number of mDC1 and mDC2 cells. However, within the patient cohort, those patients that had higher than the median number of mDC1 cells lived longer. These data suggest that mDC1 cells contribute to patient survival likely via promoting protective immunity. This is supported by further data showing that patients whose DCs maintained their ability to increase CD80 in response to activation demonstrated increased survival. The data also imply that rescuing this DC subset using immunotherapy might prolong patient life expectancy. The case studies presented in this thesis indicate that some recovery of circulating DC numbers is possible through surgery. Whilst these results are very limited as only a few patients were investigated, murine studies have demonstrated increased efficacy of combination chemotherapy and immunotherapy following surgical debulking of a tumour (Broomfield et al., 2005). It is possible that improved DC function after surgery contributed to this effect by promoting immunological memory via chemotherapy-induced apoptosis of residual tumour cells. However, the case studies suggested that any surgically-induced DC benefit is lost once radiotherapy is used.

In the absence of mesothelioma or any other serious disease, elderly-derived circulating monocytes maintained their ability to respond to GM-CSF and IL-4 and differentiate into MoDCs similar to those from younger donors. In contrast, subtle differences were seen in the responses of elderly-derived MoDCs to maturation signals; i.e. no obvious age-related global loss of function was seen after DC maturation. For example, LPS provoked decreased expression of the maturation marker, CD83, yet expression of the co-stimulatory molecules, CD80 or CD86 were similar to those seen in younger donors. IFNγ induced increased expression of CD86 whilst CD80 expression was similar to the younger counterparts.

The presence of mesothelioma further modified the patient-derived MoDC phenotype including a significant reduction in expression of the co-stimulatory molecule, CD40 in immature MoDCs relative to healthy MoDCs. Nonetheless, their responses to LPS were similar to the healthy age-matched controls which, whilst not completely restoring CD40 expression, did increase relative to the healthy controls. This was not true for responses to the combination of LPS and IFNγ which led to decreased expression of CD86 in mesothelioma patient-derived MoDCs. Similarly, the percentage of mesothelioma patient-derived MoDCs expressing CD83 was lower than that seen in the healthy controls. However, within the patient cohort, those with higher than median expression of CD80 following IFNγ activation also lived longer. This data implies that there is a subgroup of mesothelioma patients whose DCs are not fully impaired and treating them with immunotherapy may extend survival duration.

Interestingly, when MoDCs derived from healthy young volunteers were differentiated in the presence of mesothelioma tumour conditioned media, there was evidence of premature maturation, and LPS activation did not induce further phenotypic maturation. This data suggests that the normal response for DCs following exposure to tumour-derived factors is to mature/activate. The patients' lack of a maturational response to tumour-derived factors may indicate an intrinsic defect in their precursor monocytes. This could be due to their increased age or prolonged in vivo exposure to tumour-derived factors.

Responses to activation through CD40 revealed the greatest age-related differences, with healthy elderly-derived MoDCs surpassing responses seen in young-derived MoDCs in regards to increased CD1a, CD40 and CD86 expression. In contrast, mesothelioma patient-derived MoDCs showed decreases in CD1a, CD40, CD83, CD86 and HLA-DR expression. Interestingly, patients with a higher than median percentage of MoDCs expressing CD80 following CD40L activation lived longer. These data suggest that the co-stimulatory molecule CD80 may play an important role in increasing survival, and that therapies aiming to stimulate increased expression of CD80 should be further investigated.

Interestingly, the case studies indicate that surgery may offer a limited window where expression of most surface molecules, including CD40 and CD80, were increased irrespective of the stimuli used. This could be due to decreased levels of tumour-derived suppressive factors. Whilst, the study needs to be repeated with a larger sample number, this early data suggests that surgery followed by CD40-activating therapy may lead to increased survival.

The most concerning effect of mesothelioma was loss of antigen processing function in immature MoDCs. This is a key function of immature DCs including those in the tumour microenvironment as these cells are critically required to take up tumour antigen for presentation to T cells in tumour-draining lymph nodes. This loss of antigen processing function was clearly mesothelioma specific and independent of age as immature MoDCs from healthy aged-matched individuals maintained their ability to process antigen.

Under healthy conditions in young adults, loss of antigen processing function is associated with DC maturation as they transition to a cell that presents antigen in MHC molecules to T cells. However, in mesothelioma-derived DCs, loss of antigen processing ability did not correlate with an increase in the maturation marker, CD83, suggesting a specific loss of function rather than a generalised premature maturation process. Indeed, the data showed that healthy elderly-derived MoDCs retained their ability to process antigen even following activation with LPS and/or IFNγ implying an

age-related maturation paralysis. It is unclear whether this paralysis is due to defects in the mechanisms involved in down-regulating antigen processing function such as down-regulation of TAP proteins similar to that observed in cancer patients (Whiteside et al., 2004), or if elderly-derived MoDCs adopt a macrophage-like function and maintain their ability to process and destroy pathogens (Pietschmann et al., 2000). Interestingly, patients who showed a higher than median down-regulation of antigen processing from their MoDCs following activation with LPS and IFNγ lived significantly longer. This suggests that when the normal process of down-regulation of antigen processing is retained in mesothelioma patient DCs, they mature more appropriately and likely present tumour antigens to T cells to induce activated T cells that slow tumour progression.

CD40L activation appeared to rejuvenate healthy elderly-derived MoDCs as they reduced their antigen-processing capacity and matured in a similar manner to their younger counterparts. In contrast, immature mesothelioma patient-derived MoDCs could not readily process antigen and this did not change in response to CD40L activation, likely due to low CD40 expression on immature DCs. The case studies did not reveal restoration of antigen processing ability in patient MoDCs following either surgery or radiotherapy. This data suggests that immune-based therapies that aim to restore DC antigen processing function will be critical to elicit an anti-tumour T cell immune response, and that whilst a signal delivered by CD40L can restore this function in aged DCs, it cannot do so in mesothelioma-derived DCs and more powerful or alternative signals are required, such as those delivered in combination therapies.

8.2 Summary

The major findings of this thesis can be summarised as follows:

- Healthy elderly individuals have decreased numbers of pDCs in their blood, with no change in mDC populations.
- Elderly-derived MoDCs respond poorly to activation with LPS and/or IFNγ, with decreased expression of costimulatory and maturation markers and a poor ability to down-regulate antigen processing ability.

- CD40L stimulation rescues elderly-derived MoDCs from both phenotypic and antigen processing maturational paralysis.
- Mesothelioma patients have decreased numbers of pDCs, mDC1s and mDC2s in their blood.
- Mesothelioma patient-derived MoDCs have significantly decreased expression of CD40, antigen processing ability and respond poorly to activation with LPS and/or IFNγ.

Analysis of survival data suggested that DC numbers and function may contribute to survival:

- Patients with a higher number of circulating mDC1s survived significantly longer than those with a low number.
- Patients who had a higher number of LPS + IFNγ activated MoDCs decrease their antigen processing ability survived significantly longer.
- An increase in CD80 expression on IFNγ stimulated MoDCs correlated with increased survival.
- A higher than average increase in the percentage of CD40L stimulated MoDCs expressing CD80 also correlated with increased survival.

Mesothelioma-derived factors negatively impact on DC function:

- Mesothelioma patient-derived MoDCs had a limited response to 'rescue' with CD40L, possibly due to low levels of CD40 expression at their immature status.
- A brief exposure of healthy MoDCs to mesothelioma tumour supernatants induced lasting defects in their response to stimuli.

Case studies revealed:

- Radiotherapy alone offered no recovery in the number of circulating blood DCs.
- Antigen processing capacity was further reduced following radiotherapy.
- Following radiotherapy, both immature and activated MoDCs had reduced expression of CD11c, CD83, CD86 and HLA-DR.

- Surgery improved the number of circulating blood DCs, but improvements were lost following radiotherapy.
- Neither surgery nor radiotherapy restored antigen processing ability.
- Expression of CD1a, CD11c, CD80 and CD83 was increased following surgery, but lost post radiotherapy.
- Pleural fluid contained lower numbers of mDC1s and higher numbers of pDCs than circulating blood.
- Pleural fluid MoDCs demonstrated increased activation on account of a decreased capacity to process antigen possibly and higher levels of CD1a, CD11c, CD40, CD80, CD83, CD86 and HLA-DR.

8.3 Future studies

These studies have demonstrated that a greater understanding is required on how both age and mesothelioma affects dendritic cells. Further research should investigate how aging leads to the DC maturational paralysis observed in this study. Examining ROS within DCs may be useful as studies of aged mice have shown that oxidative stress induces inadequate clearing of ROS (a hallmark of aging) leading to impairments in processing exogenous antigens (Cannizzo et al., 2012).

To understand how mesothelioma impairs the number, phenotype and function of DCs, serum and pleural fluid from patients should be exhaustively analysed for the presence/concentration of tumour-derived factors and this data correlated with DC impairment. Likely factors include TGFβ, VEGF and IL-6 as these have been previously observed in high concentration in the pleural effusions of mesothelioma patients (DeLong et al., 2005). In vitro studies such as co-culture of MoDCs with individual factors and/or blocking antibodies as well as derivation of DCs from precursor cells could then be performed to identify which factors induce DC defects.

The case studies described in this thesis indicate that surgery has the potential to improve both circulating blood DC subset numbers as well as improving the expression

of maturation and co-stimulatory molecules on blood-derived MoDCs. A larger study is required with more mesothelioma patients undergoing debulking surgery.

8.4 Conclusion

In conclusion, this study has shown specific age-related changes, such as decreased circulating pDC numbers and maturational paralysis in response to LPS and/or IFNγ activation. Further defects are seen in people with mesothelioma including decreased circulating mDCs, decreased antigen processing in immature DCs, reduced expression of surface CD40 and a subsequent poor response to CD40 activation. Whilst limited in the number of patients observed, only patients undergoing surgery showed an improvement with an increase in circulating DCs. Importantly, mesothelioma patients with higher numbers of circulating mDC1s and/or DCs that maintained one or more response to maturation signals lived longer. These data suggest that functional DCs contribute to an improved life expectancy in people with mesothelioma and that a therapy tailored to improve DC numbers and function could improve patient outcomes.

9 APPENDIX

9.1 Characteristics of Healthy Study Volunteers

	Young	Elderly	p Value
Number of subjects:	30	37	
Age range in years:	20 – 44	56 - 84	
Mean age in years:	28.77	68.46	
Standard deviation of age:	7.793	6.371	
Female gender no. (%):	21 (70)	14 (38)	0.0225
Past Medical conditions:			
None:	18 (60)	8 (22)	*0.0062
Cancer:	1 (3)	3 (8)	0.7270
Blood and Cholesterol:	4 (13)	19 (51)	*0.0069
Asthma:	5 (17)	1 (3)	0.3024
Allergy:	2 (7)	2 (5)	0.9304
Diabetes:	1 (3)	3 (8)	0.7270
Flu:	0 (0)	1 (3)	
Gout:	0 (0)	1 (3)	
Hepatitis:	0 (0)	1 (3)	
Arthritis:	0 (0)	1 (3)	
Neuropathy:	0 (0)	1 (3)	
Statement of present health:			
Excellent:	11 (37)	11 (30)	0.6228
Good:	18 (60)	22 (59)	0.9743
Fine:	1 (3)	2 (5)	0.8821
Poor:	0 (0)	0 (0)	
Medication:			
No Medication:	12 (40)	7 (19)	0.1298
Cholesterol lowering:	0 (0)	13 (35)	
Blood pressure lowering:	0 (0)	12 (32)	
Antidepressant:	2 (7)	5 (14)	0.6179

Anti-coagulant:	0 (0)	7 (19)	
NSAID:	0 (0)	2 (5)	
Anti-diabetic:	0 (0)	1 (3)	
Anti-reflux:	1 (3)	7 (19)	0.2550
Thyroid drug:	0 (0)	4 (11)	
Cardiac drug:	1 (3)	3 (8)	0.7270
Anti-osteo drug:	0 (0)	1 (3)	
Oral contraceptive:	12 (40)	0 (0)	
Inhaled corticosteroid:	2 (7)	2 (5)	
Anti-histamine:	1 (3)	0 (0)	
Smoking:			
Active:	1 (3)	0 (0)	
Social:	3 (10)	1 (3)	0.5902
Non-smoker:	26 (87)	36 (97)	0.4322
Asbestos Exposure:			
No:	29 (97)	14 (38)	***<0.0001
Unknown:	1 (3)	10 (27)	0.0858
Yes:	0 (0)	13 (35)	

P-values were determined using the two-tailed Mann-Whitney test.

9.2 Formula for the calculation of antigen processing by DQ-Ova assay

Antigen uptake = (MFI of the DQ-OVA stained 37°C sample – MFI of the unstained 37°C sample) – (MFI of the DQ-Ova stained 4°C sample – MFI of the unstained 4°C sample)

9.3 Staining panels for phenotyping of MoDCs

Flurochrome	Panel 1	Panel 2
FITC	CD14	CD14
APC-CY7	HLA-DR	HLA-DR
PE-CY5	CD1a	CD40
PE	CD80	CD86
APC	CD11c	CD83

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